IMMUNOGENIC HBc CHIMER PARTICLES STABILIZED
WITH AN N-TERMINAL CYSTEINE

CROSS-REFERENCE TO RELATED APPLICATION

This a continuation-in-part of application Serial No. 09/930,915, filed August 15, 2001, whose disclosures are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to the intersection of the fields of immunology and protein engineering, and particularly to a chimeric hepatitis B virus (HBV) nucleocapsid protein that is engineered for both enhanced stability of self-assembled particles via an N-terminal cysteine residue and the display of an immunogenic epitope.

BACKGROUND OF THE INVENTION

The family hepadnaviridae are enveloped DNA-containing animal viruses that can cause hepatitis B in humans (HBV). The hepadnavirus family includes hepatitis B viruses of other mammals, e.g., woodchuck (WHV), and ground squirrel (GSHV), and avian viruses found in ducks (DHV) and herons (HeHV). Hepatitis B virus (HBV) used herein refers to a member of the family hepadnaviridae that infects mammals, as compared to a virus that infects an avian host, unless the discussion refers to a specific example of a non-mammalian virus.

The nucleocapsid or core of the mammalian hepatitis B virus (HBV or hepadnavirus) contains a sequence of 183 or 185 amino acid residues, depending

on viral subtype, whereas the duck virus capsid contains 262 amino acid residues. Hepatitis B core protein monomers of the several hepadnaviridae self-assemble in infected cells into stable aggregates known as hepatitis B core protein particles (HBc particles). Two three-dimensional structures are reported for HBc particles. A first that comprises a minor population contains 90 copies of the HBc subunit protein as dimers or 180 individual monomeric proteins, and a second, major population that contains 120 copies of the HBc subunit protein as dimers or 240 individual monomeric proteins. These particles are referred to as T = 4 or T = 3 particles, respectively, wherein "T" is the triangulation number. These HBc particles of the human-infecting virus (human virus) are about are about 30 or 34 nm in diameter, respectively. Pumpens et al. (1995) *Intervirology*, **38**:63-74; and Metzger et al. (1998) *J. Gen. Viol.*, **79**:587-590.

Conway et al., (1997) *Nature*, **386**:91-94, describe the structure of human HBc particles at 9 Ångstrom resolution, as determined from cryo-electron micrographs. Bottcher et al. (1997), *Nature,* **386**:88-91, describe the polypeptide folding for the human HBc monomers, and provide an approximate numbering scheme for the amino acid residues at which alpha-helical regions and their linking loop regions form. Zheng et al. (1992), J. *Biol. Chem.*, **267(13)**:9422-9429 report that core particle formation is not dependent upon the arginine-rich C-terminal domain, the binding of nucleic acids or the formation of disulfide bonds based on their study of mutant proteins lacking one or more cysteines and others'

work with C-terminal-truncated proteins [Birnbaum et al., (1990) *J.Virol.* **64**, 3319-3330].

The hepatitis B nucleocapsid or viral core protein (HBc) has been disclosed as an immunogenic carrier moiety that stimulates the T cell response of an immunized host animal. See, for example, U.S. Patents No. 4,818,527, No 4,882,145 and No. 5,143,726. A particularly useful application of this carrier is its ability to present foreign or heterologous B cell epitopes at the site of the immunodominant loop that is present at about residue positions 70-90, and more usually recited as about positions 75 to 85 from the amino-terminus (N-terminus) of the protein. Clarke et al. (1991) F. Brown et al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.313-318.

During viral replication, HBV nucleocapsids associate with the viral RNA pre-genome, the viral reverse transcriptase (Pol), and the terminal protein (derived from Pol) to form replication competent cores. The association between the nucleocapsid and the viral RNA pre-genome is mediated by an arginine-rich domain at the carboxyl-terminus (C-terminus). When expressed in heterologous expression systems, such as *E.coli* where viral RNA pre-genome is absent, the protamine-like C-terminus; i.e., residues at positions 150 through 183, can bind *E.coli* RNA. Zhang et al. (1992) *JBC*, **267**(13) 9422-29.

HBcAg is a particulate protein derived from the hepatitis B virus that has been proposed as a carrier for heterologous epitopes. The relative immunogenicity of HBsAg (HBs) has been compared with HbcAg (HBc), and the ability of each to evoke immune responses in different genetic backgrounds [Milich et al., *Science*, (1986)

234(4782): p. 1398-1401]. These data emphasize the higher immunogenicity of HBc relative to HBs, and the universal responsiveness to HBc, irrespective of genetic background.

For example, HBc is more than 300 times more immunogenic than HBs in BALB/c mice; and, although both B10.S and B10.M mice are non-responders to HBs, every strain tested is responsive to HBc. These results re-emphasize the suitability of HBc as a vaccine carrier and specifically, its superiority over HBs, hence the selection of HBc as opposed to HBs to carry heterologous epitopes. These facets of HBc are thought to be important in influenza vaccine development, because they address issues of genetic restriction and inadequate antibody titers.

Another advantage of the HBc carrier is the fact that it does not require complex adjuvants for efficacy. This is due to the high inherent immunogenicity of the particle. A comparison of the immunogenicity of HBc-*P. berghei* particles showed that alum, which is approved for human use, was more effective than either IFA or CFA [Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46]. The importance of this observation is highlighted by toxicity problems associated with newer, more complex adjuvants as was recently noted in clinical trials of SKB's candidate malaria vaccine [Stoute et al., *N. Engl. J. Med.*, [1997] **336**(2): p. 86-91].

In an application as a vaccine carrier moiety, it is preferable that the HBV nucleocapsids not bind nucleic acid derived from the host. Birnbaum et al. (1990) *J.Virol.*, **64**:3319-3330 showed that the protamine-like C-terminal domain of HBV nucleocapsids could be deleted without interfering with the protein's ability to assemble into virus-

like particles. It is thus reported that proteins truncated to about position 144; i.e., containing the HBc sequence from position one through about 144, can self-assemble, whereas deletions beyond residue 139 abrogate capsid assembly [F. Birnbaum & M. Nassal (1990) *J.Virl.*, 64: 3319-30] [Seifer et al., (1995) Intervirology, **38**:47-62].

Zlotnick et al., (1997) *Proc. Natl. Acad. Sci., USA*, **94**:9556-9561 studied the assembly of full length and truncated HBc proteins in to particles. In addition to discussing full length molecules, those authors reported the preparation of a truncated protein that contained the HBc sequence from position 1 through 149 in which the cysteines at positions 48, 61 and 107 were each replaced by alanines and in which a cysteine residue was added at the C-terminus (position 150). That C-terminal mercaptan was used for linkage to a gold atom cluster for labeling in electron microscopy.

More recently, Metzger et al. (1998) *J. Gen. Viol.*, **79**:587-590 reported that the proline at position 138 (Pro-138 or P138) of the human viral sequence is required for particle formation. Those authors also reported that assembly capability of particles truncated at the carboxy-terminus to lengths of 142 and 140 residues was affected, with assembly capability being completely lost with truncations resulting in lengths of 139 and 137 residues.

Several groups have shown that truncated particles exhibit reduced stability relative to standard hepatitis B core particles [Galena et al. (1989) *J.Virol.*, **63**:4645-4652; Inada, et al. (1989) *Virus Res.*, **14**:27-48], evident by variability in

particle sizes and the presence of particle fragments
in purified preparations [Maassen et al., (1994)
Arch. Virol., **135**:131-142]. Thus, prior to the
report of Metzger et al., above, Pumpens et al.,
(1995) *Intervirology*, **38**:63-74 summarized the
literature reports by stating that the carboxy-
terminal border for HBc sequences required for self-
assembly was located between amino acid residues 139
and 144, and that the first two or three amino-
terminal residues could be replaced by other
sequences, but elimination of four or eleven amino-
terminal residues resulted in the complete
disappearance of chimeric protein in transformed *E.
coli* cells.

Recombinantly-produced hybrid HBc particles
bearing internal insertions (referred to in the art
as HBc chimeric particles or HBc chimers) containing
various inserted polypeptide sequences have been
prepared by heterologous expression in a wide variety
of organisms, including *E.coli*, *B.subtilis*, *Vaccinia*,
Salmonella typhimurium, *Saccharomyces cerevisiae*.
See, for example Pumpens et al. (1995) *Intervirology*,
38:63-74 , and the citations therein that note the
work of several research groups.

Such HBc chimers often appear to have a
less ordered structure, when analyzed by electron
microscopy, compared to particles that lack
heterologous epitopes [Schodel et al., (1994)
J.Exp.Med., **180**:1037-1046]. In some cases the
insertion of heterologous epitopes into C-terminally
truncated HBc particles has such a dramatic
destabilizing affect that hybrid particles cannot be
recovered following heterologous expression [Schodel
et al. (1994) *Infect. Immunol.*, **62**:1669-1676]. Thus,

many chimeric HBc particles are so unstable that they fall apart during purification to such an extent that they are unrecoverable or they show very poor stability characteristics, making them problematic for vaccine development.

The above Pumpens et al. (1995) *Intervirology*, **38**:63-74 report lists particle-forming chimers in which the inserted polypeptide sequence is at the N-terminus, the C-terminus and between the termini. Insert lengths reported in that article are 24 to 50 residues at the N-terminus, 7 to 43 residues internally, and 11 to 741 residues at the C-terminus.

Kratz et al., (1999) *Proc. Natl. Acad. Sci., U.S.A.*, **96**:1915-1920 recently described the *E. coli* expression of chimeric HBc particles comprised of a truncated HBc sequence internally fused to the 238-residue green fluorescent protein (GFP). This chimer contained the inserted GFP sequence flanked by a pair of glycine-rich flexible linker arms replacing amino acid residues 79 and 80 of HBc. Those particles were said to effectively elicit antibodies against native GFP in rabbits as host animals.

U.S. Patent No. 5,990,085 describes two fusion proteins formed from an antigenic bovine inhibin peptide fused into (i) the immunogenic loop between residues 78 and 79 and (ii) after residue 144 of carboxy-terminal truncated HBc. Expressed fusion proteins were said to induce the production of anti-inhibin antibodies when administered in a host animal. The titers thirty days after immunization reported in that patent are relatively low, being 1:3000-15,000 for the fusion protein with the loop insertion and 1:100-125 for the insertion after residue 144.

U.S. Patent No. 6,231,864 teaches the preparation and use of a strategically modified hepatitis B core protein that is linked to a hapten. The modified core protein contains an insert of one to about 40 residues in length that contains a chemically-reactive amino acid residue to which the hapten is pendently·linked.

Recently published WO 01/27281 teaches that the immune response to HBc can be changed from a Th1 response to a Th2 response by the presence or absence, respectively, of the C-terminal cysteine-containing sequence of the native molecule. That disclosure also opines that disulfide formation by C-terminal cysteines could help to stabilize the particles. The presence of several residues the native HBc sequence immediately upstream of the C-terminal cysteine was said to be preferred, but not required. One such alternative that might be used to replace a truncated C-terminal HBc sequence was said to include a C-terminal cysteine and an optional sequence that defines an epitope from other than HBc.

Published PCT application WO 01/98333 teaches the deletion of one or more of the four arginine repeats present at the C-terminus of native HBc, while maintaining the C-terminal cysteine residue. That application also teaches that the deleted region can be replaced by an epitope from a protein other than HBc so that the HBc portion of the molecule so formed acts as a carrier for the added epitope.

Published PCT applications corresponding to PCT/US01/25625 and PCT/US01/41759 of the present inventor teach that stabilization of C-terminally truncated HBc particles can be achieved through the use of one or more

added cysteine residues in the chimer proteins from which the particles are assembled. Those added cysteine residues are taught to be at or near the C-terminus of the chimeric protein.

A structural feature whereby the stability of full-length HBc particles could be retained, while abrogating the nucleic acid binding ability of full-length HBc particles, would be highly beneficial in vaccine development using the hepadnaviral nucleocapsid delivery system. Indeed, Ulrich et al. in their recent review of the use of HBc chimers as carriers for foreign epitopes [*Adv. Virus Res.*, **50**: 141-182 (1998) Academic Press] note three potential problems to be solved for use of those chimers in human vaccines. A first potential problem is the inadvertent transfer of nucleic acids in a chimer vaccine to an immunized host. A second potential problem is interference from preexisting immunity to HBc. A third possible problem relates to the requirement of reproducible preparation of intact chimer particles that can also withstand long-term storage.

The above four published PCT applications appear to contain teachings that can be used to overcome over come the potential problems disclosed by Ulrich et al. As disclosed hereinafter, the present invention provides another HBc chimer that provides unexpectedly high titers of antibodies against influenza, and in one aspect also provides a solution to the problems of HBc chimer stability as well as the substantial absence of nucleic acid binding ability of the construct. In addition, a contemplated recombinant chimer exhibits minimal, if

any, antigenicity toward preexisting anti-HBc antibodies.

The above particle instability findings related to N-terminal truncated HBc chimer molecules notwithstanding, Neirynck et al., (October 1999) *Nature Med.*, **5(10)**:1157-1163 reported that particle formation occurred on *E. coli* expression of a HBc chimer that contained the N-terminal 24-residue portion of the influenza M2 protein fused at residue 5 to full length HBc.

The previously discussed use of hybrid HBc proteins with truncated C-termini for vaccine applications offers several advantages over their full-length counterparts, including enhanced expression levels and lack of bound *E.coli* RNA. However, C-terminally truncated particles engineered to display heterologous epitopes are often unstable, resulting in particles that either fail to associate into stable particulate structures following expression, or that readily dissociate into non-particulate structures during and/or following purification. Such a lack of stability is exhibited by particles comprised of chimeric HBc molecules that are C-terminally truncated to HBc position 149 and also contain the above residues 1-24 of the influenza A M2 protein.

Others have reported that in wild type hepadnaviral core antigens a cysteine residue upstream of the HBcAg start codon is directly involved in the prevention of particle formation [Schodel et al. (Jan. 15, 1993) *J. Biol. Chem.*, **268(2)**:1332-1337; Wasenauer et al. (Mar. 1993) *J. Virol.*, **67(3)**:1315-1322; and Nassal et al. (Jul. 1993) *J. Virol.*, **67(7)**:4307-4315]. All three groups

reported that in wild type HBeAg, the cysteine residue at position -7 of the pre-core sequence, which is present when the core gene is translated from an upstream initiator methionine at position -30, is responsible for preventing particle formation and therefore facilitating the transition from particulate HBcAg to secreted, non-particulate HBeAg.

Based upon the above three publications, one would expect the inclusion of one or more cysteine residues at a position prior to that of the initiator methionine of HBc; i.e., at a residue position of less than one relative to the N-terminus of the sequence of SEQ ID NO:1, to actually destabilize hybrid particles rather than stabilize them. As will be seen from the discussion that follows, the present invention provides results that are contrary to those expectations.

BRIEF SUMMARY OF THE INVENTION

The present invention contemplates a recombinant hepadnavirus nucleocapsid protein; i.e., a hepatitis B core (HBc) chimeric protein [or chimer hepatitis B core protein molecule or HBc chimer molecule or just chimer] that self-assembles into particles after expression in a host cell. A recombinant chimer hepatitis B core (HBc) protein molecule up to about 515 amino acid residues in length is contemplated. That chimer molecule

(a) contains an HBc sequence of at least about 125 of the N-terminal 150 amino acid residues of the HBc molecule that includes (i) the HBc sequence of residue positions 5 through about 75 and about 85 through about 140, (ii) a peptide-bonded heterologous immunogenic epitope at one or more of

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the N-terminus, in the HBc immunodominant loop or the
C-terminus of the chimer, or (iii) a heterologous
linker residue for a conjugated epitope present in
the HBc immunodominant loop,

          (b) contains one to three cysteine residues
at an amino acid position of the chimer molecule
corresponding to amino acid position -20 to about +1
from the N-terminus of the HBc sequence of SEQ ID
NO:1 [N-terminal cysteine residue(s)] in a sequence
other than that of the HBc precore sequence and zero
to about three cysteine residues toward the C-
terminus of the molecule from the C-terminal residue
of the HBc sequence and within about 30 residues from
the C-terminus of the chimer molecule [C-terminal
cysteine residue(s)].

          That chimer molecule (i) contains no more
than about 20 percent conservatively substituted
amino acid residues in the HBc sequence, (ii) self-
assembles into particles that are substantially free
of binding to nucleic acids on expression in a host
cell. The particles are more stable than are
particles formed from otherwise identical HBc chimer
molecules that are free of any above-mentioned C-
terminal cysteine residue(s) and (i) lack the N-
terminal cysteine residue(s) or (ii) in which an N-
terminal cysteine residue(s) present in a
contemplated chimer molecule is(are) replaced by
another residue.

          A preferred recombinant hepatitis B virus
core (HBc) protein chimer molecule has a length of
about 135 to about 515 amino acid residues that
contains four peptide-linked amino acid residue
sequence domains from the N-terminus that are
denominated Domains I, II, III and IV.

Domain I of that chimer molecule comprises about 71 to about 110 amino acid residues whose sequence includes (i) at least the sequence of the residues of position 5 through position 75 of HBc, (ii) one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence, and (iii) an optional heterologous immunogenic epitope containing up to about 30 amino acid residues peptide-bonded to one of HBc residues 2-4.

Domain II of that chimer molecule comprises about 5 to about 250 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which (i) zero to all residues in the sequence of HBc positions 76 to 85 are present peptide-bonded to (ii) an optionally present sequence of one to about 245 amino acid residues that are heterologous to HBc and constitute a heterologous immunogenic epitope or a heterologous linker residue for a conjugated epitope.

Chimer Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II.

Chimer molecule Domain IV comprises (i) five through fourteen residues of an HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues [C-terminal cysteine residue(s)] within about 30 residues from the C-terminus of the chimer molecule, and (iii) zero to about 100 amino acid residues in an

immunogenic sequence heterologous to HBc from position 150 to the C-terminus.

The chimer molecules (i) have an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer and (ii) self-assemble into particles on expression in a host cell. The particles are substantially free of binding to nucleic acids and are more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

It is preferred that the HBc sequence of Domain I include the residues of position 5 through position 75 along plus at least an N-terminal cysteine residue. It is further preferred that a contemplated immunogen contain not only an N-terminal cysteine residue, but also contain one cysteine residue within Domain IV as noted above that is alone or in an amino acid residue sequence heterologous to that of HBc from position 150 to the C-terminus.

The before-mentioned self-assembled chimer molecule particles are a particularly contemplated embodiment of this invention. Another embodiment comprises an inoculum or vaccine that comprises an above HBc chimer particle that is dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered in an immunogenic effective amount to an animal such as a mammal or bird, an inoculum induces antibodies that immunoreact specifically with the

chimer particle. The antibodies so induced also immunoreact specifically with (bind to) the polypeptide of the heterologous sequence and the protein from which its sequence is derived or with (to) the conjugated epitope.

The present invention has several benefits and advantages.

A particular benefit of the invention is that its use as a vaccine provides extraordinary antibody titers against pathogens such as influenza A.

An advantage of the invention is that those very high antibody titers have been produced with the aid of an adjuvant approved for use in humans.

Another benefit of the invention is that the recombinant immunogen is prepared easily and using well-known cell culture techniques.

Another advantage of the invention is that the immunogen is easily prepared using well-known recombinant techniques.

Yet another benefit of the invention is that a preferred immunogen exhibits greater stability on preparation than do other HBc chimers.

Yet another advantage of the invention is that a contemplated immunogen is substantially free of nucleic acids.

Still further benefits and advantages will be apparent to the worker of ordinary skill from the disclosure that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure

Fig.1, shown in two panels as Fig. 1A and
Fig. 1B, provides an alignment of six published
sequences for mammalian HBc proteins from six
viruses. The first (SEQ ID NO:1), human viral
sequence is of the *ayw* subtype and was published in
Galibert et al. (1983) *Nature*, **281**:646-650; the
second human viral sequence (SEQ ID NO:2), of the *adw*
subtype, was published by Ono et al. (1983) *Nucleic
Acids Res.*, **11(6):** 1747-1757; the third human viral
sequence (SEQ ID NO:3), is of the *adw2* subtype and
was published by Valenzuela et al., Animal Virus
Genetics, Field et al. eds., Academic Press, New York
(1980)pages 57-70; the fourth human viral sequence
(SEQ ID NO:4), is of the *adyw* subtype that was
published by Pasek et al. (1979) *Nature*, **282**:575-579;
the fifth sequence (SEQ ID NO:5), is that of the
woodchuck virus that was published by Galibert et al.
(1982) *J. Virol.*, 41:51-65; and the sixth mammalian
sequence, (SEQ ID NO:6), is that of the ground
squirrel that was published by Seeger et al. (1984)
J. Virol.,51:367-375.

Fig. 2 shows the modifications made to
commercial plasmid vector pKK223-3 in the preparation
of plasmid vector pKK223-3N used herein for
preparation of recombinant HBc chimers. The modified
sequence (SEQ ID NO:7) is shown below the sequence of
the commercially available vector (SEQ ID NO:8). The
bases of the added NcoI site are shown in lower case
letters and the added bases are shown with double
underlines, whereas the deleted bases are shown as
dashes. The two restriction sites present in this
segment of the sequence (NcoI and HindIII) are
indicated.

Fig. 3 is an analytical size exclusion chromatography elution profile for ICC-1603 particles in which absorbance at 280 nm is shown on the ordinate and time in seconds is shown on the abscissa.

Fig. 4 is an analytical size exclusion chromatography elution profile for ICC-1590 particles as discussed for Fig. 3.

Fig. 5 is an analytical size exclusion chromatography elution profile for ICC-1560 particles as discussed for Fig. 3.

Fig. 6 is an analytical size exclusion chromatography elution profile for ICC-1605 particles as discussed for Fig. 3.

Fig. 7 is an analytical size exclusion chromatography elution profile for ICC-1604 particles as discussed for Fig. 3.

Fig. 8 is an analytical size exclusion chromatography elution profile for ICC-1438 particles as discussed for Fig. 3.

Fig. 9 is an analytical size exclusion chromatography elution profile for ICC-1492 particles as discussed for Fig. 3.

Fig 10 is a photograph of an SDS-PAGE analysis under reducing conditions following particle preparation that shows the ICC-1438 monomer construct was unstable after aging (Lane 2) as compared to the ICC-1492 construct (Lane 3), with HBc-149 (Lane 1), ICC-1475 (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls.

Fig. 11, taken from PCT/US01/25625 (ICC-102.2) illustrates a reaction scheme (Scheme 1) that shows two reaction sequences for (I) forming an activated carrier for pendently linking a hapten to a

chimeric hepatitis B core protein (sm-HBc) particle
using sulpho-succinimidyl 4-(N-maleimidomethyl)-
cyclohexane 1-carboxylate (sulpho-SMCC), and then
(II) linking a sulfhydryl-terminated (cysteine-
terminated) hapten to the activated carrier to form a
conjugate particle. The sm-HBc particle is depicted
as a box having a single pendent amino group (for
purposes of clarity of the figure), whereas the
sulfhydryl-terminated hapten is depicted as a line
terminated with an SH group.

Definitions

Numerals utilized in conjunction with HBc
chimers indicate the position in the HBc *ayw* amino
acid residue sequence of SEQ ID NO:1 at which one or
more residues has been added to or deleted from the
sequence, regardless of whether additions or
deletions to the amino acid residue sequence are
present. Thus, HBc149 indicates that the chimer ends
at residue 149, whereas HBc149 + C150 indicates that
that same chimer contains a cysteine residue at HBc
position 150 relative to the sequence numbers of SEQ
ID NO:1.

The term "antibody" refers to a molecule
that is a member of a family of glycosylated proteins
called immunoglobulins, which can specifically bind
to an antigen.

The word "antigen" has been used
historically to designate an entity that is bound by
an antibody or receptor, and also to designate the
entity that induces the production of the antibody.
More current usage limits the meaning of antigen to
that entity bound by an antibody or receptor, whereas
the word "immunogen" is used for the entity that

induces antibody production or binds to the receptor.
Where an entity discussed herein is both immunogenic
and antigenic, reference to it as either an immunogen
or antigen is typically made according to its
intended utility.

"Antigenic determinant" refers to the
actual structural portion of the antigen that is
immunologically bound by an antibody combining site
or T-cell receptor. The term is also used
interchangeably with "epitope".

The word "conjugate" as used herein refers
to a hapten operatively linked to a carrier protein,
as through an amino acid residue side chain.

The term "conservative substitution" as
used herein denotes that one amino acid residue has
been replaced by another, biologically similar
residue. Examples of conservative substitutions
include the substitution of one hydrophobic residue
such as isoleucine, valine, leucine or methionine for
another, or the substitution of one polar residue for
another such as between arginine and lysine, between
glutamic and aspartic acids or between glutamine and
asparagine and the like.

The term "corresponds" in its various
grammatical forms as used in relation to peptide
sequences means the peptide sequence described plus
or minus up to three amino acid residues at either or
both of the amino- and carboxy-termini and containing
only conservative substitutions in particular amino
acid residues along the polypeptide sequence.

The term "Domain" is used herein to mean a
portion of a recombinant HBc chimer molecule that is
identified by (i) residue position numbering relative
to the position numbers of HBcAg subtype *ayw* as

reported by Galibert et al., (1979) *Nature*, 281:646-650 (SEQ ID NO:1). The polypeptide portions of at least chimer Domains I, II and III are believed to exist in a similar tertiary form to the corresponding sequences of naturally occurring HBcAg.

As used herein, the term "fusion protein" designates a polypeptide that contains at least two amino acid residue sequences not normally found linked together in nature that are operatively linked together end-to-end (head-to-tail) by a peptide bond between their respective carboxy- and amino-terminal amino acid residues. The fusion proteins of the present invention are HBc chimer molecules that induce the production of antibodies that immunoreact with a polypeptide that corresponds in amino acid residue sequence to the polypeptide portion of the fusion protein.

The phrase "hepatitis B" as used here refers in its broadest context to any member of the family of mammalian hepadnaviridae, as discussed before.

The words "polypeptide" and "peptide" are used interchangeably throughout the specification and designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids. Polypeptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms that are salts. It is well understood in the art that amino acid residue sequences contain acidic and basic groups, and that the particular ionization state exhibited by the peptide is dependent on the pH value of the surrounding medium when the peptide is in solution, or that of the medium from which it was

obtained if the peptide is in solid form. Thus,
"polypeptide" or its equivalent terms is intended to
include the appropriate amino acid residue sequence
referenced. A peptide or polypeptide is always shown
herein from left to right and in the direction from
amino-terminus (N-terminus) to carboxy-terminus (C-
terminus).

The term "residue" is used interchangeably
with the phrase amino acid residue. All amino acid
residues identified herein are in the natural or L-
configuration. In keeping with standard polypeptide
nomenclature, [*J. Biol. Chem.*, 243, 3557-59 (1969)],
abbreviations for amino acid residues are as shown in
the following Table of Correspondence.

TABLE OF CORRESPONDENCE

1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
Z	Glx	L-glutamic acid

		or
		L-glutamine
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
B	Asx	L-aspartic acid
		or
		L-asparagine
C	Cys	L-cysteine

DETAILED DESCRIPTION OF THE INVENTION

The present invention contemplates a
chimeric hepadnavirus nucleocapsid protein; i.e., a
recombinant hepatitis B core (HBc) protein, that is
engineered to (a) display an immunogenic B cell or T
cell epitope, or a linker for attachment of an
immunogenic B cell or T cell epitope, (b) exhibit
enhanced stability on formation when present in a
self-assembled particle, as well as exhibit (c) a
substantial absence of nucleic acid binding as a
self-assembled particle. A contemplated HBc chimer
is truncated at the C-terminus of the molecule
relative to a native HBc molecule.

Thus, the chimeric protein displays one or
more heterologous immunogenic epitopes at the N-
terminus, in the HBc immunogenic (immunodominant)
loop or C-terminus, or a linker for such a B cell or
T cell epitope in the immunogenic loop. The chimeric
protein contains a cysteine residue at or near the N-
terminus that confers enhanced stability on formation
to the self-assembled particles. The chimeric
protein is sufficiently free of arginine and or

lysine residues downstream of (toward the carboxy-terminus from) HBc residue position 149 so that the self-assembled particles are substantially free of nucleic acid binding.

For ease of discussion, contemplated chimer sequences and sequence position numbers referred to herein are based on the sequence and position numbering of the human hepatitis B core protein of subtype *ayw* [Galibert et al., (1979) *Nature*, **281**:646-650] that is shown in SEQ ID NO:1. It is to be understood, however, that in view of the great similarity between the mammalian hepadnavirus capsid protein sequences and similar particle formation exhibited by those proteins, which are well-known to skilled workers, a discussion regarding human HBc subtype *ayw* is also applicable to subtype *adw*, as well as the woodchuck and ground squirrel proteins. As a consequence of those great similarities, HBc sequences are recited generally herein as a "HBc" sequence, unless otherwise stated.

In one embodiment, a contemplated HBc chimer is up to about 515 residues in length and contains

(a) an HBc sequence of at least about 125 of the N-terminal 150 amino acid residues of the HBc molecule that includes (i) the HBc sequence of residue positions 5 through about 75 and about 85 through about 140, (ii) a peptide-bonded heterologous immunogenic epitope at one or more of the N-terminus, in the HBc immunodominant loop or the C-terminus of the chimer, or (iii) a heterologous linker residue for a conjugated epitope present in the HBc immunodominant loop, and

(b) one to three cysteine residues at an
amino acid position of the chimer molecule
corresponding to amino acid position -20 to about +1
from the N-terminus of the HBc sequence of SEQ ID
NO:1 [N-terminal cysteine residue(s)] in a sequence
other than that of the HBc precore sequence and zero
to about three cysteine residues toward the C-
terminus of the molecule from the C-terminal residue
of the HBc sequence and within about 30 residues from
the C-terminus of the chimer molecule [C-terminal
cysteine residue(s)].

That chimer molecule (i) contains no more
than about 20 percent conservatively substituted
amino acid residues in the HBc sequence, (ii) self-
assembles into particles that are substantially free
of binding to nucleic acids on expression in a host
cell. The particles are more stable on formation
than are particles formed from otherwise identical
HBc chimer molecules that are free of any above-
mentioned C-terminal cysteine residue(s) and (i) lack
the N-terminal cysteine residue(s) or (ii) in which
an N-terminal cysteine residue(s) present in a
contemplated chimer molecule is (are) replaced by
another residue.

A contemplated chimer molecule contains a
cysteine residue at a position of about -20 to about
+1 relative to the N-terminus of HBc as is
illustrated in Fig. 1 and SEQ ID NO:1. The concept
of a negative amino acid position is usually
associated with a leader sequence such as the precore
sequence of HBc. That concept is used similarly here
in that one can simply align a given chimer molecule
sequence with that of SEQ ID NO:1 to determine the
position of the chimer that corresponds to that of

the starting methionine residue of position +1 of
HBc. Inasmuch as amino acid residue sequences are
normally shown from left to right and in the
direction from N-terminus to C-terminus, any aligned
chimer molecule residue to the left of the position
occupied by the HBc start methionine has a negative
position. A contemplated cysteine residue can occur
at a position about twenty residues to the left of
the aligned start methionine of HBc to the position
corresponding to that start methionine.

In one aspect, a preferred HBc chimer has a
sequence of about 135 to about 515 L-α-amino acid
residues and contains four serially peptide-linked
domains; i.e., Domains I, II, III and IV. Those four
domains are linked together in the same manner as are
native proteins; i.e., they are peptide-bonded to
each other, as compared to polypeptides that contain
residues of other than α-amino acids and therefore
cannot form peptide bonds, those that contain D-amino
acid residues, or oligopeptide conjugates in which
two or more polypeptides are operatively linked
through an amino acid residue side chain. A
contemplated chimeric HBc protein can therefore be
prepared by expression using the usual methods of
recombinant technology.

Domain I of that chimer molecule comprises
about 71 to about 110 amino acid residues whose
sequence includes (i) at least the sequence of the
residues of position 5 through position 75 of HBc,
(ii) one to three cysteine residues at an amino acid
position of the chimer molecule corresponding to
amino acid position -20 to about +1, and preferably
amino acid position -14 to about +1, from the N-
terminus of the HBc sequence of SEQ ID NO:1 [N-

terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence, and (iii) an optional heterologous immunogenic epitope containing up to about 30 amino acid residues peptide-bonded to one of HBc residues 2-4. That heterologous immunogenic sequence, when present, is typically an epitope used to induce an immune response for a vaccine or inoculum.

Domain II of that chimer molecule comprises about 5 to about 250 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which (i) zero to all residues in the sequence of HBc positions 76 to 85, and preferably at least four HBc residues, are present peptide-bonded to (ii) an optionally present sequence of one to about 245 amino acid residues that are heterologous to HBc and constitute a heterologous immunogenic epitope or a heterologous linker residue for a conjugated epitope. It is particularly preferred that the sequence of 10 residues of positions 76 trough 85 (position 76-85 sequence) be present, but interrupted by one to about 245 residues of the heterologous linker or heterologous epitope.

Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II.

Chimer molecule Domain IV comprises (i) five through fourteen residues of an HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues [C-terminal cysteine residue(s)] within about 30 residues from the C-terminus of the chimer molecule, and (iii) zero to about 100 amino acid residues in an

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immunogenic sequence heterologous to HBc from position 150 to the C-terminus. Preferably, Domain IV contains a sequence of zero to about 50 amino acid residues in a sequence heterologous to HBc, and more preferably that sequence is zero to about 25 residues. Domain IV also preferably contains one C-terminal cysteine residue.

The chimer molecules (i) have an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer and (ii) self-assemble into particles on expression in a host cell. The particles are substantially free of binding to nucleic acids and are more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

In one aspect, a contemplated chimer molecule contains a heterologous epitope at the N-terminus peptide-bonded to one of HBc residues 2-5. In another aspect, a contemplated chimer molecule contains a heterologous epitope or a heterologous linker residue for an epitope peptide-bonded near the middle of the molecule located between HBc residues 76 and 85 in the immunodominant loop. In a further aspect, a heterologous epitope is located at the C-terminal portion of the chimer molecule peptide-bonded to one of HBc residues 136-149. In yet other aspects, two or three heterologous epitopes are present at the above locations, or one or two heterologous epitopes are present along with a

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heterologous linker residue for an epitope. Each of those chimer molecules also contains an N-terminal cysteine residue(s), as discussed before. Specific examples of several of these chimer molecules and their self-assembled particles are discussed hereinafter.

As already noted, a contemplated HBc chimer molecule can contain about 135 to about 515 amino acid residues. In some preferred embodiments, HBc residue 4 is present, whereas residues 2-5 are present in other preferred embodiments, so that Domain I can begin at HBc residue 4 or 2 and continue through residue 75; i.e., the HBc residue at HBc position 75. Residue 1 is methionine, the amino acid of the DNA start codon. It is preferred that the native methionine that is normally present at position 1 of HBc be absent so that only one start signal is present in the encoding DNA or NA.

The heterologous immunogenic epitope that can be present in Domain I or in the immunodominant loop of Domain II preferably contains about 15 to about 50 residues, although an epitope as short as about 6 amino acid residues can induce and be recognized by antibodies and T cell receptors and is therefore useful. It is preferred that all of the residues of Domain II from position 76 through position 85 are present, although interrupted by one or more other residues. Domain II must contain at least four residues, that can have any sequence that does not interfere with expression or use, but those residues are preferably part of the sequence between the residues of positions 75 and 85.

Domain III contains HBc residues 86 through 135 peptide-bonded to residue 85.

Domain IV contains a sequence of at least five residues that are comprised of (i) a sequence of the residues of HBc positions 136 through 140, and preferably through 149, peptide-bonded to residue 135, (ii) zero to three cysteines residues and (iii) optionally can contain a sequence of a heterologous epitope of up to about 100 residues, particularly when the HBc sequence ends at residue 140, although a shorter sequence of up to about 25 residues is more preferred. That Domain IV heterologous sequence is heterologous to the sequence of HBc and is other than a sequence of HBc from position 150 to the HBc C-terminus. The heterologous sequence, when present in Domain IV, is preferably a T cell epitope, but can also be a B cell epitope as are usually present in one or the other of Domains I and II.

Domain IV can also contain zero to three cysteine residues and those Cys residues are present within about 30 residues of the carboxy-terminus (C-terminus) of the chimer molecule. Preferably, one cysteine (Cys) residue is present, and that Cys is preferably present as the carboxy-terminal (C-terminal) residue, unless a T cell epitope is present as part of Domain IV. When such a T cell epitope is present, the preferred Cys is preferably within the C-terminal last five residues of the HBc chimer.

In one embodiment, a particularly preferred chimer contains two heterologous epitopes. Those two heterologous epitopes are present in Domains I and II, or II and IV, or I and IV. One of the two heterologous epitopes is preferably a B cell epitope in some embodiments. In other embodiments, one of the two heterologous epitopes is a T cell epitope. More preferably, one of the two heterologous epitopes

is a B cell epitope and the other is a T cell epitope. In addition, a plurality of B cell epitopes can be present at the B cell epitope location and a plurality of T cell epitopes can be present at the T cell epitope location.

In the embodiments in which the chimer molecule contains a heterologous epitope in Domain II, it is preferred that that the sequence contain one or more B cell epitopes, that the HBc sequence between amino acid residues 76 and 85 be present, but interrupted by the heterologous epitope(s), and that the chimer further include one or more T cell epitopes in Domain IV peptide-bonded to one of HBc residues 140-149.

This same preference holds for those chimer molecules in which the heterologous linker residue for a conjugated epitope is present in Domain II, thereby providing one or more heterologous epitopes in Domain II, with residues 76 and 85 present, but interrupted by the heterologous linker residue, with a T cell epitope being present peptide-bonded to one of HBc residues 140-149. The particles formed from such chimer molecules typically contain a ratio of conjugated epitope to C-terminal peptide-bonded T cell epitope of about 1:4 to 1:1, with a ratio of about 1:2 being common.

In an illustrative structure of an above-described chimer molecule, a heterologous linker residue for a conjugated epitope is present in Domain II and a T cell epitope is present in Domain IV, with no additional B cell epitope being present in Domain II. Such a chimer exhibits immunogenicity of the T cell epitope, while exhibiting minimal, if any, HBc antigenicity as measured by binding of anti-loop

monoclonal antibodies in an ELISA assay as discussed
hereinafter.

A preferred contemplated HBc chimer
molecule contains a sequence of about 135 to about
515 residues. A preferred HBc chimer molecule
containing two heterologous epitopes of preferred
lengths of about 15 to about 50 residues each and a
preferred HBc portion length of about 140 to about
149 residues has a sequence length of about 170 to
about 250 amino acid residues. Particularly
preferred chimer molecules continuing two
heterologous epitopes have a length of about 180 to
about 210 residues. It is to be understood that a
wide range of chimer molecule lengths is contemplated
in view of the variations in length of the N- and C-
terminal HBc portions and differing lengths of the
several contemplated epitopes that can be inserted in
the immunogenic loop.

A contemplated recombinant protein, after
expression in a host cell, self-assembles to form
particles that are substantially free of binding to
nucleic acids. The contemplated HBc chimer particles
are generally spherical in shape and are usually
homogeneous in size for a given preparation. These
chimeric particles thus resemble native HBc particles
that have a similar shape and size and can be
recovered from infected persons.

A contemplated chimer particle comprises
previously discussed chimer molecules. More broadly,
such a chimer particle comprises a chimeric C-
terminal truncated HBc protein that has a sequence of
at least about 125 of the N-terminal 150 residues and
contains (i) a heterologous epitope peptide-bonded to
one or more of the N-terminus, C-terminus or the

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immunodominant loop, or a heterologous linker residue for an epitope in the immunodominant loop, and (ii) one to three N-terminal cysteine residues and zero to three C-terminal cysteine residues as previously described, and at least a 5 HBc residue sequence from position 135.

A contemplated particle is sufficiently free of arginine and/or lysine residues in Domain IV so that the self-assembled particles are substantially free of nucleic acid binding and exhibits a 280/260 absorbance ratio of about 1.2 to about 1.7, as discussed herein after. Thus, a contemplated chimeric protein is free of the HBc sequence between positions 150 and 183.

The presence of the above-discussed N-terminal cysteine residue(s) provides an unexpected enhancement of the ability of the chimer molecules to form stable immunogenic particles (discussed hereinafter). Thus, a contemplated HBc chimer particle immunogen tends to form particles that stay together upon collection and initial purification as measured by analytical size exclusion chromatography, whose details are discussed hereinafter.

Contemplated particles are more stable upon formation than are particles formed from otherwise identical HBc chimer molecules that (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue and are also are free of any above-mentioned C-terminal cysteine residue(s). In some instances, particles do not form unless an N-terminal cysteine is present. Examples of enhanced

stabilities for both types of sequences are
illustrated in the Examples that follow.

A contemplated particle containing a N-
terminal cysteine residue is also typically prepared
in greater yield than is a particle assembled from a
chimer molecule lacking a N-terminal cysteine. This
increase in yield can be seen from the mass of
particles obtained or from analytical gel filtration
analysis using Superose® 6 HR as discussed
hereinafter.

The substantial freedom of nucleic acid
binding exhibited by contemplated particles can be
readily determined by a comparison of the absorbance
of the particles in aqueous solution measured at both
280 and 260 nm; i.e., a 280/260 absorbance ratio.
The contemplated particles do not bind substantially
to nucleic acids that are oligomeric and/or polymeric
DNA and RNA species originally present in the cells
of the organism used to express the protein. Such
nucleic acids exhibit an absorbance at 260 nm and
relatively less absorbance at 280 nm, whereas a
protein such as a contemplated chimer absorbs
relatively less at 260 nm and has a greater
absorbance at 280 nm.

Thus, recombinantly expressed HBc particles
or chimeric HBc particles that contain the arginine-
and lysine-rich sequence at residue positions 150-183
(or 150-185) sometimes referred to in the art as the
protamine region exhibit a ratio of absorbance at 280
nm to absorbance at 260 nm (280/260 absorbance ratio)
of about 0.8. On the other hand, particles
sufficiently free of arginine and lysine residues in
Domain IV so that the self-assembled particles are
substantially free of nucleic acid binding such as

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particles that are free of the arginine-rich nucleic
acid binding region of naturally occurring HBc like
as those that contain fewer than three arginine or
lysine residues or mixtures thereof adjacent to each
other, or those having a native or chimeric sequence
that ends at about HBc residue position 140 to
position 149, exhibit a 280/260 absorbance ratio of
about 1.2 to about 1.6. A more typical 280/260
absorbance ratio is about 1.4 to about 1.6. This
range is due in large part to the number of aromatic
amino acid residues present in Domains II and IV of a
given chimeric HBc particle.

Domain I of a contemplated chimeric HBc
protein constitutes an amino acid residue sequence of
HBc beginning with at least amino acid residue
position 5 through position 75, and Domain III
constitutes a HBc sequence from position 86 through
position 137. The sequences from any of the
mammalian hepadnaviruses can be used for either of
Domains I and III, and sequences from two or more
viruses can be used in one chimer. Preferably, and
for ease of construction, the human *ayw* sequence is
used through out the chimer.

HBc chimers having a Domain I that contains
more than a deletion of the first three amino-
terminal (N-terminal) residues have been reported to
result in the complete disappearance of HBc chimer
protein in *E. coli* cells. Pumpens et al., (1995)
Intervirology, **38**:63-74. On the other hand, a recent
study in which an immunogenic 23-mer polypeptide from
the influenza M2 protein was fused to the HBc N-
terminal sequence reported that the resultant fusion
protein formed particles when residues 1-4 of the
native HBc sequence were replaced. Neirynck et al.

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(October 1999) *Nature Med.,* **5(10)**:1157-1163. Thus, the art teaches that particles can form when an added amino acid sequence is present peptide-bonded to one of residues 2-4 of HBc, whereas particles do not form if no additional sequence is present and more than residues 1-3 are deleted from the N-terminus of HBc.

An N-terminal epitope sequence peptide-bonded to one of the first five N-terminal residues of HBc can contain a single cysteine residue or a sequence of up to about 30 residues that are heterologous to HBc. The one to three cysteine residues can be present at a convenient location in the sequence, but are typically near the C-terminus of the added sequence so that the added N-terminal cysteine residue(s) are at a position of about -20 to about +1, and more preferably at a position of about -14 to about +1, relative to the HBc N-terminus as shown in SEQ ID NO:1. Exemplary sequences include a B cell or T cell epitope such as those discussed and illustrated hereinafter (Tables A and B, respectively), the 23-mer polypeptide from the influenza M2 protein of Neirynck et al., above, that includes two cysteine residues, and variants of that sequence containing at least about 6 residues, a sequence of another (heterologous) protein such as β-galactosidase as can occur in fusion proteins as a result of the expression system used, or another hepatitis B-related sequence such as that from the Pre-S1 or Pre-S2 regions or the major HbsAg immunogenic sequence.

Domain II is a sequence of about 5 to about 250 amino acid residues. Of those residues, zero (none), and preferably at least 4 residues, and more preferably at least 8, constitute portions of the HBc

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sequence at positions 76 to 85, and one to about 245
residues, and preferably one to about 50 residues are
heterologous (foreign) to HBc. Thus, at least HBc
residues 75 and 85 are present in Domains I and II,
respectively. Those heterologous residues constitute
(i) a heterologous linker residue for a epitope such
as a B cell or T cell epitope or (ii) a heterologous
B or T cell epitope that preferably contains 6 to
about 50, more preferably about 15 to about 50, and
most preferably about 20 to about 30 amino acid
residues, and are positioned so that they are
peptide-bonded between zero, or more preferably at
least 4, to all of the residues of positions 76 to 85
of the HBc sequence. Heterologous B cell epitopes
are preferably linked at this position by the linker
residue or are peptide-bonded into the HBc sequence,
and use of a B cell epitope is discussed
illustratively hereinafter.

Those preferred at least 4 HBc residues can
be all in one sequence such as residues 82-85, or can
be split on either side of (flank) the heterologous
residue(s) as where residues 76-77 and 84-85 are
present or where residues 76 and 83-85 are present.
More preferably, Domain II contains at least 8
residues of the HBc sequence from residue 76 to 85.
Most preferably, the sequence of all 10 residues of
positions 76 to 85 are present in the chimer.

The one to about 245 residues added to the
HBc loop sequence is(are) heterologous to a HBc
sequence. A single added heterologous residue is a
heterologous linker residue for a B cell epitope as
discussed before. The longer sequences, typically at
least 6 amino acid residues long to about 50 amino
acid residues long and more preferably about 15 to

about 50 residues in length, as noted before, are in a sequence that comprises a heterologous immunogen such as a B cell or T cell epitope, except for heterologous residues encoded by restriction sites.

       Exemplary peptide B cell epitopes useful for both linkage to the linker residue after expression of a contemplated chimer and for expression within a HBc chimer at one or more of the N-terminus, within the immunogenic loop or at the C-terminus of the chimer are illustrated in Table A, below, along with the common name given to the gene from which the sequence is obtained, the literature or patent citation for published epitopes, and SEQ ID NO.

Table A

B Cell Epitopes

Organism	Gene	Sequence	Citation*	SEQ ID NO
Streptococcus pneumoniae				
	PspA1			
		KLEELSDKIDELDAE QKKYDEDQKKTEE-	1	9
	PsP2	KAALEKAASEEM- DKAVAAVQQA	1	10
Cryptosporidium parvum				
	P23			
		QDKPADAPAAEAPA- AEPAAQQDKPADA	2	11
HIV	GP120			
		RKRIHIGPGR- AFYITKN	3	12
Foot-and-mouth virus	VP1			
		YNGECRYNRNA- VPNLRGDLQVL- AQKVARTLP	4	13

Organism	Protein	Sequence		
Influenza Virus A8/PR8	HA	YRNLLWLTEK	8	14
Type A (A8/PR8/34)	M2	SLLTEVETPIR-NEWGCRCNGSSD	29	15
		SLLTEVETPIR-NEWGCRCNDSSD	29	16
		SLLTEVETPIR-NEWGARANDSSD		17
		EQQSAVDADDS-HFVSIELE	35	18
		SLLTEVETPIR- SLLTEVETPIR-NEWGSRSNDSSD		19
		SLLTEVETPIR-NEWGSRCNDSSD		20
		SLLTEVETPIR-NEWGCRSNDSSD		21
		SLLTEVETPIR-NEWGCRANDSSD		22
		SLLTEVETPIR-NEWGARCNDSSD		23
		MSLLTEVETPIR-NEWGCRCNDSSD		24
		MSLLTEVETPIR-NEWGSRSNDSSD		25
		MGISLLTEVETPIR-NEWGCRCNDSSD-ELLGWLWGI		26
		MSLLTEVETPIR-NEWGARANDSSD		27
		MSLLTEVETPIR-NEWGCRANDSSD		28
		MSLLTEVETPIR-NEWGARCNDSSD		29
		MSLLTEVETPIR-NEWGCRSNDSSD		30
		MSLLTEVETPIR-NEWGSRCNDSSD		31
		$X_1X_2X_3X_4X_5X_6X_7X_8$-TPIRNE$X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}$-$X_{21}X_{22}X_{23}X_{24}$		32
Type B	NB	NNATFNYTNVNPISHIR		271
Yersinia pestis	V Ag	DILKVIVDSMNHH-GDARSKLREELAE-LTAELKIYSVIQA-EINKHLSSSGTIN-IHDKSINLMDKNL-YGYTDEEIFKASA-EYKILEKMPQTTI-QVDGSEKKIVSIK-DFLGSENKRTGAL-GNLKNSYSYNKDN-NELSHFATTCSD	9	33

Haemophilus

influenza	pBOMP	CSSSNNDAA-GNGAAQFGGY	10	34
		NKLGTVSYGEE		35
		NDEAAYSKN-RRAVLAY		36
Moraxella catarrhalis	copB	LDIEKDKKK-RTDEQLQAE-LDDKYAGKGY	11	37
		LDIEKNKKK-RTEAELQAE-LDDKYAGKGY		38
		IDIEKKGKI-RTEAELLAE-LNKDYPGQGY		39
Porphyromonas gingivalis	HA	GVSPKVCKDVTV-EGSNEFAPVQNLT	12	40
		RIQSTWRQKTV-DLPAGTKYV		41
Trypanosoma cruzi		KAAIAPAKAAA-APAKAATAPA	14	42
Plasmodium falciparum	CS	$(NANP)_4$	24	43
		NANPNVDP-$(NANP)_3$NVDP		44
		NANPNVDP-$(NANP)_3$		45
		$(NANP)_3$NVDPNANP		46
		NANPNVDP-$(NANP)_3$NVDPNANP		47
		NPNVDP$(NANP)_3$NV		48
		NPNVDP-$(NANP)_3$NVDP		49
		NPNVDP$(NANP)_3$-NVDPNA		50
		NVDP$(NANP)_3$NV		51
		NVDP$(NANP)_3$NVDP		52
		NVDP$(NANP)_3$-NVDPNA		53
		DP$(NANP)_3$NV		54
		DP$(NANP)_3$NVDP		55
		DP$(NANP)_3$-NVDPNA		56
vivax	CS	GDRADGQPAG-DRADGQPAG	20	57

		RADDRAAGQP-AGDGQPAG		58
		ANGAGNQPG-ANGAGDQPG		59
		ANGADNQPG-ANGADDQPG	27	60
		ANGAGNQPG-ANGADNQPG		61
		ANGAGNQPG-ANGADDQPG		62
		APGANQEGGAA-APGANQEGGAA	28	63
		ANGAGNQPGAN-GAGDQPGANGA-DNQPGANGADD-QPG		.64
berghi	CS	DPPPPNPN-DPPPPNPN	2	65
yoelli	CS	(QGPGAP)$_4$		66
Streptococcus sobrinus	AgI/II	KPRPIYEA-KLAQNQK	16	67
		AKADYEAK-LAQYEKDL		68
Shigella flexneri	Invasin	KDRTLIEQK	18	69
Respiratory syncitia virus (RSV)	G	CSICSNNPT-CWAICK	19	70
Entamoeba histolytica	lectin	VECASTVCQNDN-·SCPIIADVEKCNQ	21	71
Schistosoma japonicum	para	DLQSEISLSLE-NGELIRRAKSA-ESLASELQRRVD	22	72
Schistosoma mansoni	para	DLQSEISLSLE-NSELIRRAKAA-ESLASDLQRRVD	22	73
Bovine Inhibin α_C subunit		STPPLPWPW-SPAALRLLQ-RPPEEPAA	30	74

Ebola Virus

    membrane-anchored glycoprotein

	ATQVEQHHRR-TDNDSTA	31	75
	HNTPVYKLD-ISEATQVE	31	76
	GKLGLITNTI-AGVAVLI	31	77

Escherichia coli

ST	CCELCCYPACAGCN	33	78
	NTFYCCELCC-YPACAGCN	33	79
	SSNYCCELCC-YPACAGCN	33	80

Alzheimer's disease

β-Amyloid	DAEFRHDSGYE-VHHQKLVFFAE-DVGSNKGAIIG-LMVGGVVIA	34	81
	DAEFRHDSGYE-VHHQKL		82
	EDVGSNKGAII		83
	DAEFRHDSGYE-VHHQKLVFFAE-DVGSNKGAIIG		84

Neisseria meningitidis

PorA	YVAVENGVAKKVA	85
	HFVQQTPKSQPTLVP	86
	HVVVNNKVATHVP	87
	PLQNIQPQVTKR	88
	AQAANGGAASGQVKVTKVTKA	89
	YVDEQSKYHA	90
	HFVQNKQNQPPTLVP	91
	KPSSTNAKTGNKVEVTKA	92
	YWTTVNTGSATTTTFVP	93
	YVDEKKKMVHA	94
	HYTRQNNADVFVP	95
	YYTKDTNNLTLVP	96
	PPQKNQSQPVVTKA	97
	PPSKGQTGNKVTKG	98
	PPSKSQPQVKVTKA	99
	QPQTANTQQGGKVKVTKA	100
	QPQVTNGVQGNQVKVTKA	101

	QPSKAQGQTNNQVKVTKA	102
	PPSSNQGKNQAQTGNTVTKA	103
	PPSKSQGKTGNQVKVTKA	104
	PPSKSQGTNNNQVKVTKA	105
	PPSKSQPGQVKVTKVTKA	106
	QLQLTEQPSSTNGQTGNQVKVT-KA	107
	QLQLTEAPSKSQGAASNQVKVT-KA	108
	SAYTPAHVYVDNKVAKHVA	109
	SAYTPAHFVQNKQNNNPTLVP	110
	VEGRNYQLQLTE	111
	PAQNSKSAYTPA	112
	QLQLTEPPSKNQAQTQNKVTKA	113
	GRDAFELFLLGSGSDE	114
	RHANVGRDAFELFLLGSGSDEA-	
	KGTDPLKNH	115
	GRDAFNLFLLGRIGDDDE	116
	GRNAFELFLIGSATSDQ	117
	QVKVTKAKSRIRTKI	118
	TLVPAVVGKPGSD	119
NspA	HAKASSSLGSAKGFSPR	120
	TRYKNYKAPSTDFKL	121
	SLNRASVDLGGSDSFSQT	122
	GKVNTVKNVRSGELSAGVRVK	123
	GKVNTVKNVRSGELSVGVRVK	124

Immunoglobulin E

	APEWPGSRDKRTL	272
	EDGQVMDVD	273
	STTQEGEL	274
	GHTFEDSTKK	275
	GGGHFPPT	276
	PGTINI	277
	FTPPT	278
	INHRGYWV	279
	GEFCINHRGYWVCGDPA	280
	MAPEWPGSRDKRTL	281
	MEDGQVMDVD	282
	MSTTQEGEL	283
	MGHTFEDSTKK	284
	MGGGHFPPT	285
	MPGTINI	286

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*Citations to published epitopes are provided following Table B.

In the above influenza A M2 sequence of SEQ ID NO: 32,

residues X_1 through X_8 are absent or present, and when present are the residues naturally present in the M2 protein sequence that are methionine, serine, leucine, leucine, threonine, glutamic acid, valine, and glutamic acid, respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X with a higher subscript number up to 8 is also present,

residues X_{15} and X_{16} are present or absent, and when present are tryptophan and glycine, respectively,

residues X_{17} and X_{19} are present or absent, and when present are independently cysteine, serine, or alanine,

residue X_{18} is present or absent, and when present is arginine, and

residues X_{20} through X_{24} are present or absent, and when present are the residues naturally present in the M2 protein sequence that are asparagine, aspartic acid, serine, serine and aspartic acid respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X residue with a lower subscript number down to 15 is also present.

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The remaining residues of Domain II that are present on either side of the heterologous residue or sequence are the residues of HBc position 76 to position 85. Thus, in a typical example, where residues 78 through 82 have been replaced, the chimer sequence in Domain II is 76 through 77, followed by restriction site-encoded residues, the heterologous immunogenic (epitope) sequence, further restriction site-encoded residues, and then HBc sequence 84 through 85. A typical exemplary sequence of a chimer prepared by an insertion strategy between residues 78 and 79 is that of HBc from position 2 through 78, followed by restriction site-encoded residues, the heterologous immunogenic sequence, further restriction site-encoded residues and HBc sequence 79 through 85. The sequence of other contemplated chimers through Domains I and II should be apparent from these illustrations and those that follow and need not be enumerated.

It has been found that a short hydrophilic peptide containing a plurality of glycine residues and having a length of about 5 to about 9 residues peptide-bonded at the C-terminus of an above-noted *Neisseria meningitidis* B cell epitope sequence can assist in the expression of a chimeric particle containing that sequence. One useful short peptide is that disclosed in Karpenko et al., *Amino Acids* (2000) 18:329-337, having the sequence GSGDGEGG of SEQ ID NO:125.

As already noted, a heterologous linker for a conjugated epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85. As was the case for the heterologous epitope, the HBc sequence of residues 76 to 85 is

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preferably present, but interrupted by the heterologous linker for a conjugated epitope. This chimer preferably includes the HBc sequence of position 4 through at least position 140, plus a cysteine residue near the N-terminus of the chimer protein. More preferably, the HBc sequence of positions 1 through 149 are present, but interrupted between residues 76 and 85 by the heterologous linker for a conjugated epitope, and the chimer molecule contains a C-terminal cysteine.

The heterologous linker for a conjugated epitope is most preferably a lysine (K) residue. Glutamic or aspartic acid, tyrosine and cysteine residues can also be used as linker residues, as can tyrosine and cysteine residues. It is noted that more than one linker can be present such as a sequence of three lysines, but such use is not preferred because heterogeneous conjugates can be formed from such use in which the conjugated hapten is bonded to one linker in a first chimer and to a different linker in a second chimer molecule. U.S. Patent No. 6,231,864 B1 discloses HBc chimer molecules containing one or more linking residues, but lacking a stabilizing N-terminal cysteine residue.

It is also noted that a heterologous epitope sequence present in a contemplated HBc chimer can also be separated from the HBc sequence residues by a "flexible linker arm" on one or both sides of (flanking) the heterologous immunogenic (epitope) sequence. This is particularly the case where the heterologous immunogenic sequence is greater than about 30 amino acid residues long. Exemplary flexible linker arm sequences typically contain about

4 to about 10 glycine residues that are thought to
permit the inserted sequence to "bulge" outwardly
from the otherwise bulging loop sequence and add
further stability to the construct.   Illustrative
flexible linker arm sequences are disclosed in Kratz
et al. (March 1999) *Proc. Natl. Acad. Sci., U.S.A.*,
96:1915-1920 and are exemplified by the amino acid
residue sequences:

<div align="center">

GGGGSGGGGT   SEQ ID NO:126

GGGGSGGGG   SEQ ID NO:127

</div>

As was noted previously, Domain III
constitutes the sequence of HBc from position 86
through position 135.   Consequently, the sequence of
the illustrative chimers discussed above for Domains
I and II, can be extended so that the first-discussed
chimer has the sequence of HBc from position 84
through position 140, and the second-discussed chimer
has the sequence of HBc from position 79 through
position 140.

Domain IV is a sequence that (i) includes a
HBc sequence from position 136 through 140 and
optionally through position 149, (ii) contains zero
up to three cysteine residues, and (iii) up to about
100 amino acid residues in a sequence heterologous to
HBc at position 150 to the C-terminus, with the
proviso that Domain IV contain at least 5 amino acid
residues of the HBc sequence from position 136
through 140.   The Domain IV sequence heterologous to
HBc more preferably contains up to about 50 amino
acid residues, and most preferably contains up to
about 25 residues.   The Domain IV sequence can thus

be substantially any sequence, except the C-terminal HBc sequence from position 150 to the C-terminus.

The length of the Domain IV sequence can be five residues; i.e., the residue of position 136 through 140, up to about 100 amino acid residues including up to a total of three cysteines, with the length being sufficient so that a contemplated chimeric protein has a total length of about 135 to about 515 residues, and more preferably up to about 460 residues, and most preferably up to about 435 amino acid residues. Where an epitope is peptide-bonded to Domains I or II contains up to about 30 or about 50 residues, respectively, as is preferred for those epitopes, more preferred lengths of the chimer molecule, including the Domain IV epitope, are about 175 to about 240 residues. Particularly preferred chimer molecules containing two heterologous epitopes have a length of about 190 to about 210 residues. Freedom of the resulting particle from nucleic acid-binding is determined by determination of the 280/260 absorbance ratio as discussed previously.

The Domain IV sequence can include zero up to three Cys residues. When present, it is preferred that the one or more Cys residues be at or within about five amino acid residues of the C-terminus of the chimeric protein molecule. In addition, when more than one Cys residue is present in a Domain IV sequence, it is preferred that those Cys residues be adjacent to each other.

It is preferred that the Domain IV sequence constitute a T cell epitope, a plurality of T cell epitopes that are the same or different or an additional B cell epitope for the organism against which a contemplated chimer is intended to be used as

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an immunogen. Exemplary Domain IV T cell epitope sequences are provided in Table B, below, as in Table A, with illustrative added C-terminal cysteine residues underlined, .

Table B

T Cell Epitopes

Organism	Gene	Sequence*	Citation	SEQ ID NO
HIV	P24	GPKEPFRDY- VDRFYK<u>C</u>	3	128
Corynebacterium diptheriae	toxin	FQVVHNSYN- RPAYSPG<u>C</u>	5	129
Borrelia burgdorferi	ospA	VEIKEGTVTLKRE- IDKNGKVTVSL<u>C</u>	6	130
		TLSKNISKSG- EVSVELND<u>C</u>	7	131
Influenza Virus A8/PR8	HA	SSVSSFERFE<u>C</u>	8	132
		LIDALLGDP<u>C</u>	32	133
		TLIDALLG<u>C</u>	32	134
Trypanosoma cruzi		SHNFTLVASVII- EEAPSGNT<u>C</u>	13	135
Plasmodium falciparum	MSP1	SVQIPKVPYPNGIVY<u>C</u>	15	136
		DFNHYYTLKTGLEAD<u>C</u>		137
		PSDKHIEQYKKI- KNSISC	23	138
		EYLNKIQNSLST- EWSPCSVT	26	139
P. vivax		YLDKVRATVGTE- WTPCSVT		140
P. yoelii		EFVKQISSQLTE- EWSQCSVT		141

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Streptococcus				
sobrinus	AgI/II			
		KPRPIYEAKL-AQNQKC	16	142
		AKADYEAKLA-QYEKDLC		143
LCMV (lymphocytic				
choriomeningitis virus)				
	NP	RPQASGVYM-GNLTAQC	17	144
Clostridium				
tetani	tox			
		QYIKANSKFIG-ITELC	20	145
Neisseria meningitidis				
	PorB	AIWQVEQKASIAGTDSGWC		146
		NYKNGGFFVQYGGAYKRHC		147
		HNSQTEVAATLAYRFGNVC		148
	PorB	TPRVSYAHGFKGLVDDADC		149
		RFGNAVPRISYAHGFDFIC		150
		AFKYARHANVGRNAFELFC		151
		SGAWLKRNTGIGNYTQINAC		152
		AGEFGTLRAGRVANQC		153
		IGNYTQINAASVGLRC		154
		GRNYQLQLTEQPSRTC		155
		SGSVQFVPAQNSKSAC		156
		HANVGRDAFNLFLLGC		157
		LGRIGDDDEAKGTDPC		158
		SVQFVPAQNSKSAYKC		159
		NYAFKYAKHANVGRDC		160
		AHGFDFIERGKKGENC		161
		GVDYDFSKRTSAIVSC		162
		HDDMPVSVRYDSPDFC		163
		RFGNAVPRISYAHGFDFIERGKKGENC		164
		NYAFKYAKHANVGRDAFNLFLLGC		165
		SGAWLKRNTGIGNYTQINAASVGLRC		166
		SGSVQFVPAQNSKSAYTPAC		167
	OpaB	TGANNTSTVSDYFRNRITC		168
		IYDFKLNDKFDKFKPYIGC		169
	Opa-5d	LSAIYDFKLNDKFKPYIGC		170
	Opac	NGWYINPWSEVKFDLNSRC		171

*Underlined C (C) is not from the native sequence.

Citations:

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3. US No. 5,639,854.

4. US No. 4,544,500.

5. EPO 399001 B1.

6. Bockenstedt et al. (1996) *J. Immunol.*, 157, 12:5496.

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22. Nara et al. (1997) *Vaccine 15*, 1:79.

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25. Schodel et al. (1994) *J. Exper. Med.*, 180:1037.

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31. Wilson et al. (2000) *Science*, 287:1664-1666.

32. Brown et al. (1993) *J. Virol.*, 67(5):2887-2893.

33. U.S. No. 4,886,663.

34. Schenk et al. (Jul 8, 1999) *Nature*, 400(6740):116-117.

35. Slepushkin et al. (1995) *Vaccine*, 13(15):1399-1402.

The amino acid sequence of HBc from residue position 4 through at least position 140 is preferably present in a contemplated chimer molecule and particle. The sequence from position 2 through position 149 is more preferably present. A B cell epitope is preferably present between residues 76 and 85 and at least a single cysteine residue at or near the N-terminus in Domain I as already noted, a T cell epitope that can include a cysteine residue can also be present as a C-terminal addition to the HBc

sequence. A contemplated recombinant HBc chimer is substantially free of bound nucleic acid. A contemplated chimer particle that contains an added Cys residue at or near the N-terminus of the molecule is also more stable after formation than is a similar particle that does not contain that added Cys.

A contemplated recombinant HBc chimer molecule is typically present and is used as a self-assembled particle. These particles are comprised of 180 to 240 chimer molecules (90 or 120 dimer pairs), usually 240 chimer molecules, that separate into protein molecules in the presence of disulfide reducing agents such as 2-mercaptoethanol, and the individual molecules are therefore thought to be bound together into the particle primarily by disulfide bonds.

Although not wishing to be bound by theory, it is believed that the observed enhanced stability and in some cases enhanced expression for a contemplated HBc chimer is due to the formation of an N-terminal cystine disulfide bond between chimer protein molecules of the particles. Regardless of whether present as a cysteine or a cystine, the N-terminal cysteine(s) residue is referred to as a cysteine inasmuch as that is the residue coded-for by the codon present in the nucleic acid from which the protein and assembled particle is expressed.

These particles are similar to the particles observed in patients infected with HBV, but these particles are non-infectious. Upon expression in various prokaryotic and eukaryotic hosts, the individual recombinant HBc chimer molecules assemble in the host into particles that can be readily

harvested from the host cells, and purified, if
desired.

As noted before, the HBc immunodominant
loop is usually recited as being located at about
positions 75 to 85 from the amino-terminus (N-
terminus) of the intact protein. The heterologous B
cell epitope-containing sequence of Domain II is
placed into that immunodominant loop sequence. That
placement substantially eliminates the HBc
immunogenicity of the HBc loop sequence, while
presenting the heterologous sequence or linker
residue in an extremely immunogenic position in the
assembled chimer particles.

In addition to the before-discussed N- and
C-truncations, insertion of various epitopes and
spacers, a contemplated chimer molecule can also
contain conservative substitutions in the amino acid
residues that constitute HBc Domains I, II, III and
IV. Conservative substitutions are as defined
before. An illustrative conservative substitution is
seen in the replacement of residues at positions 2
and 3 (aspartic acid and isoleucine; DI) by glutamic
acid and leucine (EL) residues that are encoded by an
EcoRI restriction site used to add nucleic acids that
code for a desired N-terminal epitope, including an
N-terminal cysteine residue.

More rarely, a "nonconservative" change,
e.g., replacement of a glycine with a tryptophan is
contemplated. Analogous minor variations can also
include amino acid deletions or insertions, or both.
Guidance in determining which amino acid residues can
be substituted, inserted, or deleted without
abolishing biological activity or particle formation
can be found using computer programs well known in

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the art, for example LASERGENE software (DNASTAR
Inc., Madison, Wis.)

The HBc portion of a chimer molecule of the
present invention; i.e., the portion having the HBc
sequence that has other than a sequence or residue of
an added epitope, linker, flexible linker arm or
heterologous residue(s) that are a restriction enzyme
artifact, most preferably has the amino acid residue
sequence at positions 2 through 149 of subtype *ayw*
that is shown in Fig. 1 (SEQ ID NO:1), less any
portion or portions of the subtype *ayw* sequence that
are absent because of truncation at one or both
termini. Somewhat less preferred are the
corresponding amino acid residue sequences of
subtypes *adw*, *adw2* and *adyw* that are also shown in
Fig. 1 (SEQ ID NOs:2, 3 AND 4). Less preferred still
are the sequences of woodchuck and ground squirrel at
aligned positions 2 through 149 that are the last two
sequences of Fig 1 (SEQ ID NOs:5 and 6). As noted
elsewhere, portions of different sequences from
different mammalian HBc proteins can be used together
in a single chimer.

When the HBc portion of a chimer molecule
of the present invention as above described has other
than a sequence of a mammalian HBc molecule
corresponding to positions 2 through 149, no more
than about 20 percent of the amino acid residues are
substituted as compared to SEQ ID NO:1 from position
2 through 149. It is preferred that no more than
about 10 percent, and more preferably no more than
about 5 percent, and most preferably no more than
about 3 percent of the amino acid residues are
substituted as compared to SEQ ID NO:1 from position
2 through 149.

A contemplated chimer of 149 HBc residues
can therefore contain up to about 30 residues that
are different from those of SEQ ID NO:1 at positions
2 through 149, and preferably about 15 residues.
More preferably, about 7 or 8 residues are different
from the *ayw* sequence (SEQ ID NO:1) at residue
positions 2-149, and most preferably about 4 or 5
residues are different.   Substitutions, other than in
the immunodominant loop of Domain II or at the
termini, are preferably in the non-helical portions
of the chimer molecule and are typically between
residues 2 to about 15 and residues 24 to about 50 to
help assure particle formation.   See, Koschel et al.,
J. Virol., **73(3)**:2153-2160 (Mar. 1999).

Where a HBc sequence is truncated at the C-
terminus beyond position 149 or at the N-terminus, or
contains one or more deletions in the immunogenic
loop, the number of substituted residues is
proportionally different because the total length of
the sequence is less that 149 residues.   Deletions
elsewhere in the molecule are considered conservative
substitutions for purposes of calculation.

Chimer Preparation

A contemplated chimeric HBc immunogen is
typically prepared using the well-known techniques of
recombinant DNA technology.   Thus, sequences of
nucleic acid that encode particular polypeptide
sequences are added to and deleted from the precursor
sequence that encodes HBc to form a nucleic acid that
encodes a contemplated chimer.

An illustrative contemplated chimeric
immunogen typically utilizes a cysteine residue
present in the influenza A M2 sequence as the N-

terminal cysteine. Primers for the preparation of
such chimer molecules by *in vitro* mutagenesis of a
polynucleotide encoding an HBc molecule are discussed
hereinafter. When a cysteine-containing M2
polypeptide epitope is not present at the N-terminus,
the N-terminal cysteine can be provided by *in vitro*
mutagenesis using a primer that encodes just a
cysteine-containing portion of the M2 polypeptide or
a simple N-terminal start sequence such as Met-Cys-
or Met-Gly-Cys-.

I THINK I NEED SOME MORE WORDS FOR PREP OF N-TERM CYS
-CONTAINING SEQUENCES.

        Either of two strategies is preferred for
placing the heterologous epitope sequence into the
loop sequence. The first strategy is referred to as
replacement in which DNA that codes for a portion of
the immunodominant loop is excised and replaced with
DNA that encodes a heterologous epitope such as a B
cell sequence. The second strategy is referred to as
insertion in which a heterologous epitope is inserted
between adjacent residues in the loop.
        Site-directed mutagenesis using the
polymerase chain reaction (PCR) is used in one
exemplary replacement approach to provide a chimeric
HBc DNA sequence that encodes a pair of different
restriction sites, e.g. EcoRI and SacI, one near each
end of the immunodominant loop-encoding DNA.
Exemplary residues replaced are 76 through 81. The
loop-encoding section is excised, a desired sequence
that encodes the heterologous B cell epitope is
ligated into the restriction sites and the resulting

DNA is used to express the HBc chimer. See, for
example, Table 2 of Pumpens et al., (1995)
Intervirology, **38**:63-74 for exemplary uses of this
technique.

Alternatively, a single restriction site or
two sites can be encoded into the region by site-
directed mutagenesis, the DNA cut with a restriction
enzyme to provide "sticky" ends. The sticky ends can
be used for ligation or made blunt with endonuclease
and a blunt-ended heterologous DNA segment ligated
into the cut region. Examples of this type of
sequence replacement into HBc can be found in the
work reported in Schodel et al., (1991) F. Brown et
al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York, pp.319-325; Schodel et
al., *Behring Inst. Mitt.*, 1997(98): p. 114-119 and
Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-
4, the latter two papers discussing the preparation
of vaccines against *P. yoelii* and *P. berghei*,
respectively.

The insertion position within the HBc
immunogenic loop and the presence of loop residues
can be of import to the activity of the immunogen.
Thus, as is illustrated published PCT applications
PCT/US01/25625 and PCT/US01/41759, placement of a
malarial B cell epitope between HBc residue positions
78 and 79 provides a particulate immunogen that is
ten to one thousand times more immunogenic than
placement of the same immunogen in an excised and
replaced region between residues 76 and 81. In
addition, placement of the same malarial immunogen
between residues 78 and 79 as compared to between
residues 77 and 78 provided an unexpected enhancement
in immunogenicity of about 15-fold.

Insertion is therefore generally preferred. In an illustrative example of the insertion strategy, site-directed mutagenesis is used to create two restriction sites adjacent to each other and between codons encoding adjacent amino acid residues, such as those at residue positions 78 and 79. This technique adds twelve base pairs that encode four amino acid residues (two for each restriction site) between formerly adjacent residues in the HBc loop.

Upon cleavage with the restriction enzymes, ligation of the DNA coding for the heterologous B cell epitope sequence and expression of the DNA to form HBc chimers, the HBc loop amino acid sequence is seen to be interrupted on its N-terminal side by the two residues encoded by the 5' restriction site, followed toward the C-terminus by the heterologous B-cell epitope sequence, followed by two more heterologous, non-loop residues encoded by the 3' restriction site and then the rest of the loop sequence. This same strategy can be used for insertion into Domain I of an N-terminal cysteine or N-terminal sequence as was reported in Neirynck et al., (October 1999) *Nature Med.*, **5(10)**:1157-1163 or for insertion into Domain IV of a T cell epitope or one or more cysteine residues. A similar strategy using an insertion between residues 82 and 83 is reported in Schodel et al., (1990) F. Brown et al. eds., *Vaccines 90*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.193-198.

More specifically, a DNA sequence that encodes a C-terminal truncated HBc sequence (HBc149) is engineered to contain adjacent EcoRI and SacI sites between residues 78 and 79. Cleavage of that DNA with both enzymes provides one fragment that

encodes HBc positions 1-78 3'-terminated with an EcoRI sticky end, whereas the other fragment has a 5'-terminal SacI sticky end and encodes residues of positions 79-149. Ligation of a synthetic nucleic acid having a 5' AATT overhang followed by a sequence that encodes a desired B cell epitope and a AGCT 3'overhang provides a HBc chimer sequence that encodes that B cell epitope flanked on each side by two heterologous residues [GlyIle (GI) and GluLeu (EL), respectively] between residues 78 and 79, while usually destroying the EcoRI site and preserving the SacI site.

A similar strategy for insertion of a cysteine-containing sequence in Domain IV, such as a malarial T cell epitope that contains the *P. falciparum* CS protein sequence from position 326 through position 345 and is referred to herein as PF/CS326-345 (Pf-UTC). Here, EcoRI and HindIII restriction sites are engineered into the HBc DNA sequence after amino acid residue position 149. After digestion with EcoRI and HindIII, a synthetic DNA having the above AATT 5'overhang followed by a T cell epitope-encoding sequence, one or more stop codons and a 3' AGCT overhang were ligated into the digested sequence to form a sequence that encoded HBc residues 1-149 followed by two heterologous residues (GI), the stop codon and the HindIII site.

PCR amplification using a forward primer having a SacI restriction site followed by a sequence encoding HBc beginning at residue position 79, followed by digestion with SacI and HindIII provided a sequence encoding HBc positions 79-149 plus the two added residues and the T cell epitope at the C-terminus. Digestion of the construct with SacI and

ligation provides the complete gene encoding a
desired recombinant HBc chimer immunogen having the
sequence, from the N-terminus, of HBc positions 1-78,
two added residues, the malarial B cell epitope, two
added residues, HBc positions 79-149, two added
residues, and the T cell epitope that is shown in
Fig. 2C.

Similar techniques can be used to place a
heterologous linker residue for conjugation of a B
cell epitope into the loop region sequence.
Contemplated linker residues include lysine (Lys),
which is particularly preferred, aspartic acid (Asp),
glutamic acid (Glu), cysteine (Cys) and tyrosine
(Tyr).

It is noted that the amino acid residue
sequence shown in SEQ ID NO:1 contains a Glu and an
Asp residue at positions 77 and 78. Nonetheless,
introduction of an additional, heterologous,
carboxyl-containing residue is still contemplated.
The chemical reactivity of the existing glutamic and
aspartic acids may be reduced by other factors. For
example, it is known in the art that a neighboring
proline, such as that found at position 79, can
neutralize and thereby reduce the chemical reactivity
of a proximal carboxyl group.

Here, using the first noted insertion
strategy, five heterologous residues are placed into
the loop sequence; one that is the heterologous
linker residue for conjugating a B cell epitope and
two residues adjacent on either side of that one
residue that are themselves also adjacent to loop
sequence residues and are an expression product of
the inserted restriction sites (restriction enzyme
artifacts). It is noted that one can also use site-

HBc loop sequence that encodes the heterologous
linker residue for a B cell epitope.

It is noted that the preferred use of two
heterologous residues on either side of (flanking) a
B cell or T cell epitope is a matter of convenience.
As a consequence, one can also use zero to three or
more added residues that are not part of the HBc
sequence on either or both sides of an inserted
sequence. One or both ends of the insert and HBc
nucleic acid can be "chewed back" with an appropriate
nuclease (e.g. S1 nuclease) to provide blunt ends
that can be ligated together. Added heterologous
residues that are neither part of the inserted B cell
or T cell epitopes nor a part of the HBc sequence are
not counted in the number of residues present in a
recited Domain, unless those residues are
conservative replacements for residues already
present, as where the residues GluLeu replace AspIle
in some of the constructs discussed hereinafter.

It is also noted that one can also
synthesize all or a part of a desired recombinant HBc
chimer nucleic acid using well-known synthetic
methods as is discussed and illustrated in U. S.
Patent No. 5,656,472 for the synthesis of the 177
base pair DNA that encodes the 59 residue ribulose
bis-phosphate carboxylase-oxygenase signal peptide of
Nicotiana tabacum. For example, one can synthesize
Domains I and II with a blunt or a "sticky end" that
can be ligated to Domains III and IV to provide a
construct that expresses a contemplated HBc chimer
that contains zero added residues to the N-terminal
side of the B cell epitope and zero to three added

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residues on the C-terminal side or at the Domain
II/III junction or at some other desired location.

An alternative insertion technique was
reported in Clarke et al. (1991) F. Brown et al.
eds., *Vaccines 91*, Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York, pp.313-318. Here,
taking advantage of the degeneracy of the genetic
code, those workers engineered a single restriction
site corresponding to residues 80 and 81 that encoded
the original residues present at those positions.
Their expressed HBc chimers thereby contained no
restriction site-encoded residues, and contained the
residues of the HBc loop immediately adjacent to the
inserted sequence.

A nucleic acid sequence (segment) that
encodes a previously described HBc chimer molecule or
a complement of that coding sequence is also
contemplated herein. Such a nucleic acid segment is
present in isolated and purified form in some
preferred embodiments.

In living organisms, the amino acid residue
sequence of a protein or polypeptide is directly
related via the genetic code to the deoxyribonucleic
acid (DNA) sequence of the gene that codes for the
protein. Thus, through the well-known degeneracy of
the genetic code additional DNAs and corresponding
RNA sequences (nucleic acids) can be prepared as
desired that encode the same chimer amino acid
residue sequences, but are sufficiently different
from a before-discussed gene sequence that the two
sequences do not hybridize at high stringency, but do
hybridize at moderate stringency.

High stringency conditions can be defined
as comprising hybridization at a temperature of about

50°-55°C in 6XSSC and a final wash at a temperature of 68°C in 1-3XSSC. Moderate stringency conditions comprise hybridization at a temperature of about 50°C to about 65°C in 0.2 to 0.3 M NaCl, followed by washing at about 50°C to about 55°C in 0.2X SSC, 0.1% SDS (sodium dodecyl sulfate).

A nucleic sequence (DNA sequence or an RNA sequence) that (1) itself encodes, or its complement encodes, a chimer molecule whose HBc portion from residue position 4 through 136, when present, is that of SEQ ID NOs: 1, 2, 3, 4, 5 or 6 and (2) hybridizes with a DNA sequence of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 at least at moderate stringency (discussed above); and (3) whose HBc sequence shares at least 80 percent, and more preferably at least 90 percent, and even more preferably at least 95 percent, and most preferably 100 percent identity with a DNA sequence of SEQ ID NOs: 172, 173, 174, 175, 176 and 177, is defined as a DNA variant sequence. As is well-known, a nucleic acid sequence such as a contemplated nucleic acid sequence is expressed when operatively linked to an appropriate promoter in an appropriate expression system as discussed elsewhere herein.

An analog or analogous nucleic acid (DNA or RNA) sequence that encodes a contemplated chimer molecule is also contemplated as part of this invention. A chimer analog nucleic acid sequence or its complementary nucleic acid sequence encodes a HBc amino acid residue sequence that is at least 80 percent, and more preferably at least 90 percent, and most preferably is at least 95 percent identical to the HBc sequence portion from residue position 4 through residue position 136 shown in SEQ ID NOs: 1,

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2, 3, 4, 5 or 6. This DNA or RNA is referred to
herein as an "analog of" or "analogous to" a sequence
of a nucleic acid of SEQ ID NOs: 172, 173, 174, 175,
176 and 177, and hybridizes with the nucleic acid
sequence of SEQ ID NOs: 172, 173, 174, 175,176 and
177 or their complements herein under moderate
stringency hybridization conditions. A nucleic acid
that encodes an analogous sequence, upon suitable
transfection and expression, also produces a
contemplated chimer.

Different hosts often have preferences for
a particular codon to be used for encoding a
particular amino acid residue. Such codon
preferences are well known and a DNA sequence
encoding a desired chimer sequence can be altered,
using *in vitro* mutagenesis for example, so that host-
preferred codons are utilized for a particular host
in which the enzyme is to be expressed. In addition,
one can also use the degeneracy of the genetic code
to encode the HBc portion of a sequence of SEQ ID
NOs: 172, 173, 174, 175, 176 or 177 that avoids
substantial identity with a DNA of SEQ ID Nos: 1, 2,
3, 4, 5 or 6 or their complements. Thus, a useful
analogous DNA sequence need not hybridize with the
nucleotide sequences of SEQ ID NOs: 172, 173, 174,
175, 176 or 177 or a complement under conditions of
moderate stringency, but can still provide a
contemplated chimer molecule.

A recombinant nucleic acid molecule such as
a DNA molecule, comprising a vector operatively
linked to an exogenous nucleic acid segment (e.g., a
DNA segment or sequence) that defines a gene that
encodes a contemplated chimer, as discussed above,
and a promoter suitable for driving the expression of

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the gene in a compatible host organism, is also contemplated in this invention. More particularly, also contemplated is a recombinant DNA molecule that comprises a vector comprising a promoter for driving the expression of the chimer in host organism cells operatively linked to a DNA segment that defines a gene for the HBc portion of a chimer or a DNA variant that has at least 90 percent identity to the chimer gene of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 and hybridizes with that gene under moderate stringency conditions.

Further contemplated is a recombinant DNA molecule that comprises a vector containing a promoter for driving the expression of a chimer in host organism cells operatively linked to a DNA segment that is an analog nucleic acid sequence that encodes an amino acid residue sequence of a HBc chimer portion that is at least 80 percent identical, more preferably 90 percent identical, and most preferably 95 percent identical to the HBc portion of a sequence of SEQ ID NOs: 1, 2, 3, 4, 5 or 6. That recombinant DNA molecule, upon suitable transfection and expression in a host cell, provides a contemplated chimer molecule.

It is noted that because of the 30 amino acid residue N-terminal sequence of ground squirrel HBc does not align with any of the other HBc sequences, that sequence and its encoding nucleic acid sequences and their complements are not included in the above percentages of identity, nor are the portions of nucleic acid that encode that 30-residue sequence or its complement used in hybridization determinations. Similarly, sequences that are truncated at either or both of the HBc N- and C-

termini are not included in identity calculations,
nor are those sequences in which residues of the
immunodominant loop are removed for insertion of a
heterologous epitope. Thus, only those HBc-encoding
bases or HBc sequence residues that are present in a
chimer molecule are included and compared to an
aligned nucleic acid or amino acid residue sequence
in the identity percentage calculations.

Inasmuch as the coding sequences for the
gene disclosed herein is illustrated in SEQ ID NOs:
172, 173, 174, 175, 176 and 177, isolated nucleic
acid segments, preferably DNA sequences, variants and
analogs thereof can be prepared by *in vitro*
mutagenesis, as is well known in the art and
discussed in Current Protocols In Molecular Biology,
Ausabel et al. eds., John Wiley & Sons (New York:
1987) p. 8.1.1-8.1.6, that begin at the initial ATG
codon for a gene and end at or just downstream of the
stop codon for each gene. Thus, a desired
restriction site can be engineered at or upstream of
the initiation codon, and at or downstream of the
stop codon so that other genes can be prepared,
excised and isolated.

As is well known in the art, so long as the
required nucleic acid, illustratively DNA sequence,
is present, (including start and stop signals),
additional base pairs can usually be present at
either end of the segment and that segment can still
be utilized to express the protein. This, of course,
presumes the absence in the segment of an operatively
linked DNA sequence that represses expression,
expresses a further product that consumes the enzyme
desired to be expressed, expresses a product that
consumes a wanted reaction product produced by that

desired enzyme, or otherwise interferes with expression of the gene of the DNA segment.

Thus, so long as the DNA segment is free of such interfering DNA sequences, a DNA segment of the invention can be about 500 to about 15,000 base pairs in length. The maximum size of a recombinant DNA molecule, particularly an expression vector, is governed mostly by convenience and the vector size that can be accommodated by a host cell, once all of the minimal DNA sequences required for replication and expression, when desired, are present. Minimal vector sizes are well known. Such long DNA segments are not preferred, but can be used.

DNA segments that encode the before-described chimer can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al. (1981) *J. Am. Chem. Soc.*, **103**:3185. Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. However, DNA segments including sequences discussed previously are preferred.

A contemplated HBc chimer can be produced (expressed) in a number of transformed host systems, typically host cells although expression in acellular, *in vitro*, systems is also contemplated. These host cellular systems include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g. baculovirus); plant cell systems transformed with

virus expression vectors (e.g. cauliflower mosaic
virus; tobacco mosaic virus) or with bacterial
expression vectors (e.g., Ti plasmid); or
appropriately transformed animal cell systems such as
CHO, VERO or COS cells. The invention is not limited
by the host cell employed.

DNA segments containing a gene encoding the
HBc chimer are preferably obtained from recombinant
DNA molecules (plasmid vectors) containing that gene.
Vectors capable of directing the expression of a
chimer gene into the protein of a HBc chimer is
referred to herein as an "expression vector".

An expression vector contains expression
control elements including the promoter. The chimer-
coding gene is operatively linked to the expression
vector to permit the promoter sequence to direct RNA
polymerase binding and expression of the chimer-
encoding gene. Useful in expressing the polypeptide
coding gene are promoters that are inducible, viral,
synthetic, constitutive as described by Poszkowski et
al. (1989) *EMBO J.*, **3**:2719 and Odell et al. (1985)
Nature, **313**:810, as well as temporally regulated,
spatially regulated, and spatiotemporally regulated
as given in Chua et al. (1989) *Science*, **244**:174-181.

One preferred promoter for use in
prokaryotic cells such as *E. coli* is the Rec 7
promoter that is inducible by exogenously supplied
nalidixic acid. A more preferred promoter is present
in plasmid vector JHEX25 (available from Promega
Corp., Madison WI) that is inducible by exogenously
supplied isopropyl-β-D-thiogalacto-pyranoside (IPTG).
A still more preferred promoter, the tac promoter, is
present in plasmid vector pKK223-3 and is also
inducible by exogenously supplied IPTG. The pKK223-3

plasmid can be successfully expressed in a number of *E. coli* strains, such as XL-1, TB1, BL21 and BLR, using about 25 to about 100 µM IPTG for induction. Surprisingly, concentrations of about 25 to about 50 µM IPTG have been found to provide optimal results in 2 L shaker flasks and fermentors.

Several strains of *Salmonella* such as *S. typhi* and *S. typhimurium and S. typhimurium-E. coli* hybrids have been used to express immunogenic transgenes including prior HBc chimer particles both as sources of the particles for use as immunogens and as live, attenuated whole cell vaccines and inocula, and those expression and vaccination systems can be used herein. See, U.S. Patent No. 6,024,961; U.S. Patent No. 5,888,799; U.S. Patent No. 5,387,744; U.S. Patent No. 5,297,441; Ulrich et al., (1998) *Adv. Virus Res.*, **50**:141-182; Tacket et al., (Aug 1997) *Infect. Immun.*, **65(8)**:3381-3385; Schodel et al., (Feb 1997) *Behring Inst. Mitt.*, **98**:114-119; Nardelli-Haefliger et al., (Dec 1996) *Infect. Immun.*, **64(12)**:5219-5224; Londono et al., (Apr 1996) *Vaccine,* **14(6)**:545-552, and the citations therein.

Expression vectors compatible with eukaryotic cells, such as those compatible with yeast cells or those compatible with cells of higher plants or mammals, are also contemplated herein. Such expression vectors can also be used to form the recombinant DNA molecules of the present invention. Vectors for use in yeasts such as *S. cerivisiae* or *Pichia pastoris* can be episomal or integrating, as is well known. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Normally, such vectors contain one or more convenient restriction sites for

insertion of the desired DNA segment and promoter
sequences.   Optionally, such vectors contain a
selectable marker specific for use in eukaryotic
cells.   Exemplary promoters for use in *S. cerevisiae*
include the *S. cerevisiae* phosphoglyceric acid kinase
(PGK) promoter and the divergent promoters GAL 10 and
GAL 1, whereas the alcohol oxidase gene (AOX1) is a
useful promoter for *Pichia pastoris*.

For example, to produce chimers in the
methylotrophic yeast, *P. pastoris*, a gene that
encodes a desired chimer is placed under the control
of regulatory sequences that direct expression of
structural genes in *Pichia*.   The resultant
expression-competent forms of those genes are
introduced into *Pichia* cells.

More specifically, the transformation and
expression system described by Cregg et al. (1987)
Biotechnology, **5**:479-485; (1987) *Molecular and
Cellular Biology*, **12**:3376-3385 can be used.   A gene
for a chimer V12.Pf3.1 is placed downstream from the
alcohol oxidase gene (AOX1) promoter and upstream
from the transcription terminator sequence of the
same AOX1 gene.   The gene and its flanking regulatory
regions are then introduced into a plasmid that
carries both the *P. pastoris* HIS4 gene and a *P.
pastoris* ARS sequence (Autonomously Replicating
Sequence), which permit plasmid replication within *P.
pastoris* cells [Cregg et al. (1987) *Molecular and
Cellular Biology*, **12**:3376-3385].

The vector also contains appropriate
portions of a plasmid such as pBR322 to permit growth
of the plasmid in *E. coli* cells.   The resultant
plasmid carrying a chimer gene, as well as the
various additional elements described above, is

illustratively transformed into a his4 mutant of *P. pastoris*; i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described Cregg et al. (1987) *Molecular and Cellular Biology*, **12**:3376-3385, to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the chimer protein and the production of chimer particles in *P. pastoris*.

A contemplated chimer gene can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al. (1987) *Molecular and Cellular Biology*, **12**:3376-3385.

Production of chimer particles by recombinant DNA expression in mammalian cells is illustratively carried out using a recombinant DNA vector capable of expressing the chimer gene in Chinese hamster ovary (CHO) cells. This is accomplished using procedures that are well known in the art and are described in more detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratories (1989).

In one illustrative example, the simian virus (SV40) based expression vector, pKSV-10 (Pharmacia Fine Chemicals, Piscataway, NJ), is subjected to restriction endonuclease digestion by NcoI and HindIII. A NcoI/HindIII sequence fragment that encodes the desired HBc chimer prepared as described in Example 1 is ligated into the expression plasmid, which results in the formation of a circular recombinant expression plasmid denominated pSV-Pf.

The expression plasmid pSV-Pf contains an intact *E. coli* ampicillin resistance gene. *E. coli* RR101 (Bethesda Research Laboratories, Gaithersburg, MD), when transformed with pSV-Pf, can thus be selected on the basis of ampicillin resistance for those bacteria containing the plasmid. Plasmid-containing bacteria are then cloned and the clones are subsequently screened for the proper orientation of the inserted coding gene into the expression vector.

The above obtained plasmid, pSV-Pf, containing the gene that encodes a desired HBc chimer is propagated by culturing *E. coli* containing the plasmid. The plasmid DNA is isolated from *E. coli* cultures as described in Sambrook et al., above.

Expression of a chimer is accomplished by the introduction of pSV-Pf into the mammalian cell line, e.g., CHO cells, using the calcium phosphate-mediated transfection method of Graham et al. (1973) *Virol.*, **52**:456, or a similar technique.

To help ensure maximal efficiency in the introduction of pSV-Pf into CHO cells in culture, the transfection is carried out in the presence of a second plasmid, pSV2NEO (ATCC #37149) and the cytotoxic drug G418 (GIBCO Laboratories, Grand Island, NY) as described by Southern et al. (1982) *J. Mol. Appl. Genet.*, **1**:327. Those CHO cells that are resistant to G418 are cultured, have acquired both plasmids, pSV2NEO and pSV-Pf, and are designated CHO/pSV-Pf cells. By virtue of the genetic architecture of the pSV-Pf expression vector, a chimer is expressed in the resulting CHO/pSV-Pf cells and can be detected in and purified from the cytoplasm of these cells. The resulting composition

containing cellular protein is separated on a column
as discussed elsewhere herein.

The choice of which expression vector and
ultimately to which promoter a chimer-encoding gene
is operatively linked depends directly on the
functional properties desired, e.g. the location and
timing of protein expression, and the host cell to be
transformed. These are well known limitations
inherent in the art of constructing recombinant DNA
molecules. However, a vector useful in practicing
the present invention can direct the replication, and
preferably also the expression (for an expression
vector) of the chimer gene included in the DNA
segment to which it is operatively linked.

In one preferred embodiment, the host that
expresses the chimer is the prokaryote, *E. coli*, and
a preferred vector includes a prokaryotic replicon;
i.e., a DNA sequence having the ability to direct
autonomous replication and maintenance of the
recombinant DNA molecule extrachromosomally in a
prokaryotic host cell transformed therewith. Such
replicons are well known in the art.

Those vectors that include a prokaryotic
replicon can also include a prokaryotic promoter
region capable of directing the expression of a
contemplated HBc chimer gene in a host cell, such as
E. coli, transformed therewith. Promoter sequences
compatible with bacterial hosts are typically
provided in plasmid vectors containing one or more
convenient restriction sites for insertion of a
contemplated DNA segment. Typical of such vector
plasmids are pUC8, pUC9, and pBR329 available from
Biorad Laboratories, (Richmond, CA) and pPL and
pKK223-3 available from Pharmacia, Piscataway, NJ.

Typical vectors useful for expression of genes in cells from higher plants and mammals are well known in the art and include plant vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers et al. (1987) *Meth. in Enzymol.*, **153**:253-277 and mammalian expression vectors pKSV-10, above, and pCI-neo (Promega Corp., #E1841, Madison, WI). However, several other expression vector systems are known to function in plants including pCaMVCN transfer control vector described by Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA*, **82**:58-24. Plasmid pCaMVCN (available from Pharmacia, Piscataway, NJ) includes the cauliflower mosaic virus CaMV 35S promoter.

The above plant expression systems typically provide systemic or constitutive expression of an inserted transgene. Systemic expression can be useful where most or all of a plant is used as the source to a contemplated chimer molecule or resultant particles or where a large part of the plant is used to provide an oral vaccine. However, it can be more efficacious to express a chimer molecule or particles in a plant storage organ such as a root, seed or fruit from which the particles can be more readily isolated or ingested.

One manner of achieving storage organ expression is to use a promoter that expresses its controlled gene in one or more preselected or predetermined non-photosynthetic plant organs. Expression in one or more preselected storage organs with little or no expression in other organs such as roots, seed or fruit versus leaves or stems is referred to herein as enhanced or preferential expression. An exemplary promoter that directs

expression in one or more preselected organs as
compared to another organ at a ratio of at least 5:1
is defined herein as an organ-enhanced promoter.
Expression in substantially only one storage organ
and substantially no expression in other storage
organs is referred to as organ-specific expression;
i.e., a ratio of expression products in a storage
organ relative to another of about 100:1 or greater
indicates organ specificity. Storage organ-specific
promoters are thus members of the class of storage
organ-enhanced promoters.

Exemplary plant storage organs include the
roots of carrots, taro or manioc, potato tubers, and
the meat of fruit such as red guava, passion fruit,
mango, papaya, tomato, avocado, cherry, tangerine,
mandarin, palm, melons such cantaloupe and
watermelons and other fleshy fruits such as squash,
cucumbers, mangos, apricots, peaches, as well as the
seeds of maize (corn), soybeans, rice, oil seed rape
and the like.

The CaMV 35S promoter is normally deemed to
be a constitutive promoter. However, recent research
has shown that a 21-bp region of the CaMV 35S
promoter, when operatively linked into another,
heterologous usual green tissue promoter, the rbcS-3A
promoter, can cause the resulting chimeric promoter
to become a root-enhanced promoter. That 21-bp
sequence is disclosed in U.S. Patent No. 5,023,179.
The chimeric rbcS-3A promoter containing the 21-bp
insert of U.S. Patent No. 5,023,179 is a useful root-
enhanced promoter herein.

A similar root-enhanced promoter, that
includes the above 21 bp segment is the -90 to +8
region of the CAMV 35S promoter itself. U.S. Patent

No. 5,110,732 discloses that that truncated CaMV 35S promoter provides enhanced expression in roots and the radical of seed, a tissue destined to become a root. That promoter is also useful herein.

Another useful root-enhanced promoter is the -1616 to -1 promoter of the oil seed rape (*Brassica napus L.*) gene disclosed in PCT/GB92/00416 (WO 91/13922 published Sep. 19, 1991). *E. coli* DH5.alpha. harboring plasmid pRlambdaS4 and bacteriophage lambda.beta.1 that contain this promoter were deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on Mar. 8, 1990 and have accession numbers NCIMB40265 and NCIMB40266. A useful portion of this promoter can be obtained as a 1.0 kb fragment by cleavage of the plasmid with HaeIII.

A preferred root-enhanced promoter is the mannopine synthase (mas) promoter present in plasmid pKan2 described by DiRita et al. (1987) *Mol. Gen. Genet*, **207**:233-241. This promoter is removable from its plasmid pKan2 as a XbaI-XbaII fragment.

The preferred mannopine synthase root-enhanced promoter is comprised of the core mannopine synthase (mas) promoter region up to position -138 and the mannopine synthase activator from -318 to -213, and is collectively referred to as AmasPmas. This promoter has been found to increase production in tobacco roots about 10- to about 100-fold compared to leaf expression levels.

Another root specific promoter is the about 500 bp 5' flanking sequence accompanying the hydroxyproline-rich glycopeprotein gene, HRGPnt3, expressed during lateral root initiation and reported by Keller et al. (1989) *Genes Dev., * **3**:1639-1646.

Another preferred root-specific promoter is present in the about -636 to -1 5' flanking region of the tobacco root-specific gene ToRBF reported by Yamamoto et al. (1991) *Plant Cell*, **3**:371-381. The cis-acting elements regulating expression are more specifically located by those authors in the region from about -636 to about -299 5' from the transcription initiation site. Yamamoto et al. reported steady state mRNA production from the ToRBF gene in roots, but not in leaves, shoot meristems or stems.

Still another useful storage organ-specific promoter are the 5' and 3' flanking regions of the fruit-ripening gene E8 of the tomato, *Lycopersicon esculentum*. These regions and their cDNA sequences are illustrated and discussed in Deikman et al. (1988) *EMBO J.*, **7**(11):3315-3320 and (1992) *Plant Physiol.*, **100**:2013-2017.

Three regions are located in the 2181 bp of the 5' flanking sequence of the gene and a 522 bp sequence 3' to the poly (A) addition site appeared to control expression of the E8 gene. One region from -2181 to -1088 is required for activation of E8 gene transcription in unripe fruit by ethylene and also contributes to transcription during ripening. Two further regions, -1088 to -863 and -409 to -263, are unable to confer ethylene responsiveness in unripe fruit but are sufficient for E8 gene expression during ripening.

The maize sucrose synthase-1 (Sh) promoter that in corn expresses its controlled enzyme at high levels in endosperm, at much reduced levels in roots and not in green tissues or pollen has been reported to express a chimeric reporter gene, β-glucuronidase (GUS), specifically in tobacco phloem cells that are

abundant in stems and roots. Yang et al. (1990)
Proc. Natl. Acad. Sci., U.S.A., **87**:4144-4148. This
promoter is thus useful for plant organs such as
fleshy fruits like melons, e.g. cantaloupe, or seeds
that contain endosperm and for roots that have high
levels of phloem cells.

Another exemplary tissue-specific promoter
is the lectin promoter, which is specific for seed
tissue. The lectin protein in soybean seeds is
encoded by a single gene (Le1) that is only expressed
during seed maturation and accounts for about 2 to
about 5 percent of total seed mRNA. The lectin gene
and seed-specific promoter have been fully
characterized and used to direct seed specific
expression in transgenic tobacco plants. See, e.g.,
Vodkin et al. (1983) <u>Cell</u>, **34**:1023 and Lindstrom et
al. (1990) *Developmental Genetics,* **11**:160.

A particularly preferred tuber-specific
expression promoter is the 5' flanking region of the
potato patatin gene. Use of this promoter is
described in Twell et al. (1987) *Plant Mol. Biol.,*
9:365-375. This promoter is present in an about 406
bp fragment of bacteriophage LPOTI. The LPOTI
promoter has regions of over 90 percent homology with
four other patatin promoters and about 95 percent
homology over all 400 bases with patatin promoter
PGT5. Each of these promoters is useful herein. See,
also, Wenzler et al. (1989) *Plant Mol. Biol.,* **12**:41-
50.

Still further organ-enhanced and organ-
specific promoter are disclosed in Benfey et al.
(1988) *Science,* **244**:174-181.

Each of the promoter sequences utilized is
substantially unaffected by the amount of chimer

molecule or particles in the cell. As used herein, the term "substantially unaffected" means that the promoter is not responsive to direct feedback control (inhibition) by the chimer molecules or particles accumulated in transformed cells or transgenic plant.

Transfection of plant cells using *Agrobacterium tumefaciens* is typically best carried out on dicotyledonous plants. Monocots are usually most readily transformed by so-called direct gene transfer of protoplasts. Direct gene transfer is usually carried out by electroportation, by polyethyleneglycol-mediated transfer or bombardment of cells by microprojectiles carrying the needed DNA. These methods of transfection are well-known in the art and need not be further discussed herein. Methods of regenerating whole plants from transfected cells and protoplasts are also well-known, as are techniques for obtaining a desired protein from plant tissues. See, also, U.S. Patents No. 5,618,988 and 5,679,880 and the citations therein.

A transgenic plant formed using *Agrobacterium* transformation, electroportation or other methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous chimer molecule-encoding gene segregates independently during mitosis and meiosis. A

transgenic plant containing an organ-enhanced promoter driving a single structural gene that encodes a contemplated HBc chimeric molecule; i.e., an independent segregant, is a preferred transgenic plant.

More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced chimer particle accumulation relative to a control (native, non-transgenic) or an independent segregant transgenic plant. A homozygous transgenic plant exhibits enhanced chimer particle accumulation as compared to both a native, non-transgenic plant and an independent segregant transgenic plant.

It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous (heterologous) genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a chimeric HBc molecule. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

A transgenic plant of this invention thus has a heterologous structural gene that encodes a contemplated chimeric HBc molecule. A preferred transgenic plant is an independent segregant for the added heterologous chimeric HBc structural gene and

can transmit that gene to its progeny. A more
preferred transgenic plant is homozygous for the
heterologous gene, and transmits that gene to all of
its offspring on sexual mating.

Inasmuch as a gene that encodes a chimeric
HBc molecule does not occur naturally in plants, a
contemplated transgenic plant accumulates chimeric
HBc molecule particles in a greater amount than does
a non-transformed plant of the same type or strain
when both plants are grown under the same conditions.

The phrase "same type" or "same strain" is
used herein to mean a plant of the same cross as or a
clone of the untransformed plant. Where alleic
variations among siblings of a cross are small, as
with extensively inbred plant, comparisons between
siblings can be used or an average arrived at using
several siblings. Otherwise, clones are preferred
for the comparison.

Seed from a transgenic plant is grown in
the field greenhouse, window sill or the like, and
resulting sexually mature transgenic plants are self-
pollinated to generate true breeding plants. The
progeny from these plants become true breeding lines
that are evaluated for chimeric HBc molecule particle
accumulation, preferably in the field, under a range
of environmental conditions.

A transgenic plant homozygous for chimeric
HBc molecule particle accumulation is crossed with a
parent plant having other desired traits. The
progeny, which are heterozygous or independently
segregatable for chimeric HBc molecule particle
accumulation, are backcrossed with one or the other
parent to obtain transgenic plants that exhibit
chimeric HBc molecule particle accumulation and the

other desired traits. The backcrossing of progeny with the parent may have to be repeated more than once to obtain a transgenic plant that possesses a number of desirable traits.

An insect cell system can also be used to express a HBc chimer. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) or baculovirus is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in Trichoplusia larvae.

The sequences encoding a chimer can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of chimer sequence renders the polyhedrin gene inactive and produces recombinant virus lacking coat protein. The recombinant viruses can then be used to infect, for example, *S. Frugiperda* cells or *Trichoplusia* larvae in which the HBc chimer can be expressed. E. Engelhard et al. (1994) *Proc. Natl. Acad. Sci., USA,* **91**:3224-3227; and V. Luckow, Insect Cell Expression Technology, pp. 183-218, in Protein Engineering: Principles and Practice, J.L. Cleland et al. eds., Wiley-Liss, Inc, 1996). Heterologous genes placed under the control of the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often expressed at high levels during the late stages of infection.

Recombinant baculoviruses containing the chimeric gene are constructed using the baculovirus shuttle vector system [Luckow et al. (1993) *J. Virol.,* **67**:4566-4579], sold commercially as the Bac-To-Bac™ baculovirus expression system (Life Technologies). Stocks of recombinant viruses are

prepared and expression of the recombinant protein is monitored by standard protocols (O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, W.H. Freeman and Company, New York, 1992; and King et al., The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992).

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Alternatively, synthetic linkers containing one or more restriction endonuclease sites can be used to join the DNA segment to the expression vector, as noted before. The synthetic linkers are attached to blunt-ended DNA segments by incubating the blunt-ended DNA segments with a large excess of synthetic linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase.

Thus, the products of the reaction are DNA segments carrying synthetic linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction endonuclease and ligated into an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the synthetic linker. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including New England BioLabs, Beverly, MA. A

desired DNA segment can also be obtained using PCR
technology in which the forward and reverse primers
contain desired restriction sites that can be cut
after amplification so that the gene can be inserted
into the vector. Alternatively PCR products can be
directly cloned into vectors containing T-overhangs
(Promega Corp., A3600, Madison, WI) as is well known
in the art.

The expressed chimeric protein self-
assembles into particles within the host cells,
whether in single cells or in cells within a
multicelled host. The particle-containing cells are
harvested using standard procedures, and the cells
are lysed using a French pressure cell, lysozyme,
sonicator, bead beater or a microfluidizer
(Microfluidics International Corp., Newton MA).
After clarification of the lysate, particles are
precipitated with 45 percent ammonium sulfate,
resuspended in 20 mM sodium phosphate, pH 6.8 and
dialyzed against the same buffer. The dialyzed
material is clarified by brief centrifugation and the
supernatant subjected to gel filtration
chromatography using Sepharose® CL-4B. Particle-
containing fractions are identified, subjected to
hydroxyapatite chromatography, and reprecipitated
with ammonium sulfate prior to resuspension, dialysis
and sterile filtration and storage at -70°C.

HBc Chimer Conjugates

Any hapten (immunogen) to which a B cell or
T cell response is desired can be linked to a
contemplated HBc chimer or chimer particle such as a
chimer particle containing a heterologous linker
residue such as a lysine, glutamic or aspartic acid,

cysteine or tyrosine in the loop region of Domain II
and an added cysteine residue near the N-terminus in
Domain I to form a HBc chimer conjugate. The hapten
of interest typically is a B cell immunogen. The
hapten can be a polypeptide, a protein, a
carbohydrate (saccharide; i.e., oligo- or
polysaccharide), or a non-polypeptide, non-
carbohydrate chemical such as 2,4-dinitrobenzene or a
medicament such as cocaine or nicotine. (it is thus
seen that the word "hatpen" is used herein somewhat
more broadly that is usual to include small molecules
that do not themselves induce an immune response, as
well as larger molecule such as proteins that often
can themselves induce an immune response. A HBc
chimer particle conjugate so formed is useful as an
inoculum or vaccine, as is discussed hereinafter.
Because the chimer protein self assembles upon
expression and a conjugate is formed after
expression, conjugate formation is typically done
using the assembled particles as compared to the free
protein molecules.

Methods for operatively linking individual
haptens (immunogens) to a protein or polypeptide
through an amino acid residue side chain of the
protein or polypeptide to form a pendently-linked
immunogenic conjugate, e.g., a branched-chain
polypeptide polymer, are well known in the art.
Those methods include linking through one or more
types of functional groups on various side chains and
result in the carrier protein polypeptide backbone
(here, a HBc chimer) within the particle being
pendently linked--covalently linked (coupled)-- to
the hapten but separated by at least one side chain.

Methods for linking carrier proteins to haptens using each of the above functional groups are described in Erlanger, (1980) *Method of Enzymology*, **70**:85; Aurameas *et al.*, (1978) *Scand. J. Immunol.*, Vol. 8, Suppl. 7, 7-23 and U.S. Patent No. 4,493,795 to Nestor *et al.* In addition, a site-directed coupling reaction, as described in Rodwell *et al.* (1985) *Biotech.*, **3**:889-894 can be carried out so that the biological activity of the polypeptides is not substantially diminished.

Furthermore, as is well known in the art, both the HBc protein and a polypeptide hapten can be used in their native form or their functional group content can be modified by succinylation of lysine residues or reaction with cysteine-thiolactone. A sulfhydryl group can also be incorporated into either carrier protein or conjugate by reaction of amino functional groups with 2-iminothiolane, the N-hydroxysuccinimide ester of 3-(3-dithiopyridyl)-propionate, or other reagents known in the art.

The HBc chimer or hapten can also be modified to incorporate a spacer arm, such as hexamethylenediamine or another bifunctional molecule, to facilitate the pendent linking. Such a procedure is discussed below.

Methods for covalent bonding of a polypeptide hapten are extremely varied and are well known by workers skilled in the immunological arts. For example, following U.S. Patent No. 4,818,527, m-maleimidobenzoyl-N-hydroxysuccinimide ester (ICN Biochemicals, Inc., Costa Mesa, CA) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co., Rockford, IL) is reacted with an appropriate HBc chimer to form an activated carrier.

That activated carrier is then reacted with a hapten such as a sulfhydryl-terminated hapten or a polypeptide that either contains a terminal cysteine or to which an additional amino- or carboxy-terminal cysteine residue has been added to form a covalently bonded HBc chimer conjugate. As an alternative example, the amino group of a polypeptide hapten can be first reacted with N-succinimidyl 3-(2-pyridylthio)propionate (SPDP, Pharmacia, Piscataway, NJ), and that thiol-containing polypeptide can be reacted with the activated carrier after reduction. Of course, the sulfur-containing moiety and double bond-containing Michael acceptor can be reversed. These reactions are described in the supplier's literature, and also in Kitagawa, *et al.* (1976) *J. Biochem.*, **79**:233 and in Lachmann *et al.*, in <u>1986 Synthetic Peptides as Antigens</u>, (Ciba Foundation Symposium 119), pp. 25-40 (Wiley, Chichester: 1986).

U.S. Patent No. 4,767,842 teaches several modes of covalent attachment between a carrier and polypeptide that are useful here. In one method, tolylene diisocyanate is reacted with the carrier in a dioxane-buffer solvent at zero degrees C to form an activated carrier. A polypeptide hapten is thereafter admixed and reacted with the activated carrier to form the covalently bonded HBc chimer conjugate.

Particularly useful are a large number of heterobifunctional agents that form a disulfide link at one functional group end and an amide link at the other, including N-succidimidyl-3-(2-pyridyldithio)-propionate (SPDP), discussed before that creates a disulfide linkage between itself and a thiol in either the HBc chimer or the hapten. Exemplary

reagents include a cysteine residue in a polypeptide
hapten and an amine on the coupling partner such as
the ε-amine of a lysine or other free amino group in
the carrier protein. A variety of such
disulfide/amide forming agents are known. See for
example *Immun. Rev.* (1982) **62**:185.

Other bifunctional coupling agents form a
thioether rather than a disulfide linkage. Many of
these thioether-forming agents are commercially
available and include reactive esters of
6-maleimidocaproic acid, 2-bromoacetic acid,
2-iodoacetic acid, 4-(N-maleimidomethyl)cyclohexane-
1-carboxylic acid and the like. The carboxyl groups
can be activated by combining them with succinimide
or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt.
The particularly preferred coupling agent for the
method of this invention is succinimidyl
4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)
obtained from Pierce Chemical Co., Rockford, IL. The
foregoing list is not meant to be exhaustive, and
modifications of the named compounds can clearly be
used. Fig. 11 provides a schematic representation
(Scheme 1) of the formation of a HBc activated
carrier using SMCC (I) and the subsequent reaction of
that activated carrier with a sulfhydryl-terminated
hapten (II).

A polypeptide hapten can be obtained in a
number of ways well known in the art. Usual peptide
synthesis techniques can be readily utilized. For
example, recombinant and PCR-based techniques to
produce longer peptides are useful. Because the
desired sequences are usually relatively short, solid
phase chemical synthesis is useful.

Exemplary polypeptide haptens are shown in Tables A and B hereinbefore. Each of those polypeptides can be utilized via its N-terminal amino group, or by use of an additional N-terminal cysteine that is not shown in the table.

Related chemistry is used to couple what may be called "chemical compounds" to carrier proteins. Typically, an appropriate functional group for coupling is designed into the chemical compound. An exemplary chemical hapten to which induced antibodies protect against *Streptococcus pneumoniae* is 6-*O*-phosphocholine hydroxyhexanoate. Fischer et al. (1995) *J. Immunol.*, **154**:3373-3382. The table below provides further exemplary chemical haptens.

Chemical Haptens

Chemical Hapten	Citation
piperidine N-oxide	U.S. Patent No. 5,304,252
phospholactone or lactamide	U.S. Patent No. 5,248,611
metal ion complexes	U.S. Patent No. 5,236,825
[2.2.1] or [7.2.2] bicyclic ring compounds	U.S. Patent No. 5,208,152
ionically charged hydroxyl-containing compounds	U.S. Patent No. 5,187,086
phosphonate analogs of carboxylate esters	U.S. Patent No. 5,126,258
cocaine analogs	Carrera et al., (1995) *Nature* **378**:725

There are many methods known in the art to couple carrier proteins to polysaccharides. Aldehyde groups can be prepared on either the reducing end [Anderson (1983) *Infect. Immun.*, **39**:233-238; Jennings et al. (1981) *J. Immunol.*, **127**:1011-1018; Poren et al. (1985) *Mol. Immunol.*, **22**:907-919] or the terminal end [Anderson et al. (1986) *J. Immunol.*, **137**:1181-1186; Beuvery et al. (1986) *Dev. Bio. Scand.*, **65**:197-204] of an oligosaccharide or relatively small polysaccharide, which can be linked to the carrier protein via reductive amination.

Large polysaccharides can be conjugated by either terminal activation [Anderson et al.(1986) *J. Immunol.*, **137**:1181-1186] or by random activation of several functional groups along the polysaccharide chain [Chu et al. (1983) *Infect. Immun.*, **40**:245-256; Gordon, U.S. Patent No. 4,619,828 (1986); Marburg, U.S. Patent No. 4,882,317 (1989)]. Random activation of several functional groups along the polysaccharide chain can lead to a conjugate that is highly cross-linked due to random linkages along the polysaccharide chain. The optimal ratio of polysaccharide to carrier protein depends on the particular polysaccharide, the carrier protein, and the conjugate used.

Detailed reviews of methods of conjugation of saccharide to carrier proteins can be found in Dick et al., in Contributions to Microbiology and Immunology, Vol. 10, Cruse *et al.*, eds., (S. Karger: 1989), pp. 48-114; Jennings et al., in Neoglycoconjugates: Preparation and Applications, Lee et al., eds., (Academic Press: 1994), pp. 325-371; Aplin et al., (1981) *CRC Crit. Rev. Biochem.*,

10:259-306; and Stowell et al.(1980) *Adv. Carbohydr. Chem. Biochem.*, **37**:225-281.

The carbohydrate itself can be synthesized by methods known in the art, for example by enzymatic glycoprotein synthesis as described by Witte et al. (1997) *J. Am. Chem. Soc.*, **119**:2114-2118.

Several oligosaccharides, synthetic and semi-synthetic, and natural, are discussed in the following paragraphs as examples of oligosaccharides that are contemplated haptens to be used in making a HBc conjugate of the present invention.

An oligosaccharide hapten suitable for preparing vaccines for the treatment of *Haemophilus influenza* type b (Hib) is made up of from 2 to 20 repeats of D-ribose-D-ribitol-phosphate (**I**, below), D-ribitol-phosphate-D-ribose (**II**, below), or phosphate-D-ribose-D-ribitol (**III**, below). Eduard C. Beuvery et al., EP-0 276 516-B1.
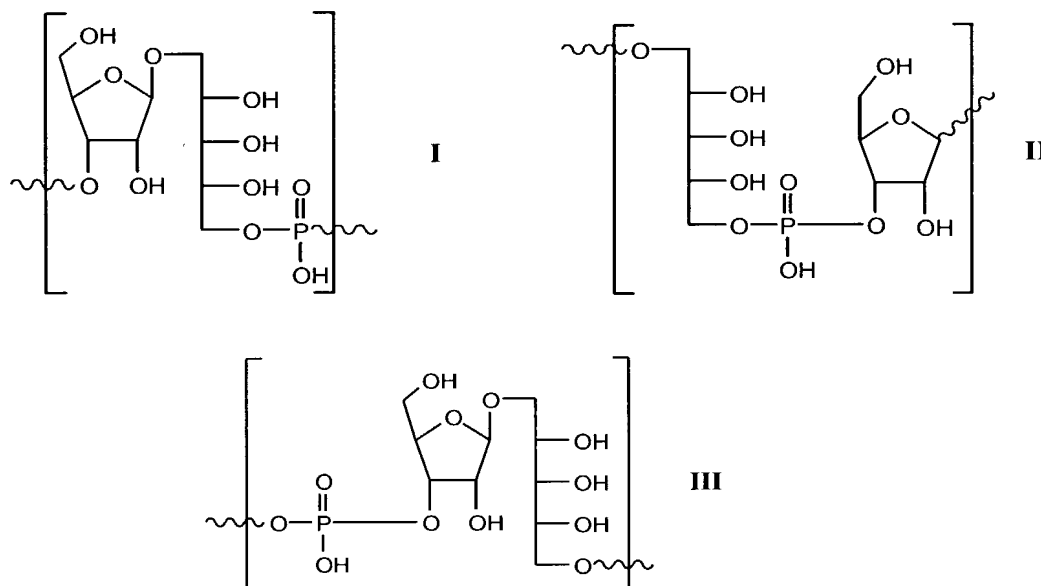
U.S. Patent No. 4,220,717 also discloses a
polyribosyl ribitol phosphate (PRP) hapten for
Haemophilus influenzae type b.

Peterson et al. (1998) *Infect. Immun.*,
66(8):3848-3855, disclose a trisaccharide hapten,
αKdo(2 8)αKdo(2 4)αKdo, that provides protection from
Chlamydia pneumoniae. *Chlamydia pneumoniae* is a
cause of human respiratory infections ranging from
pharyngitis to fatal pneumonia. Kdo is 3-deoxy-D-
manno-oct-2-ulosonic acid.

Andersson et al., EP-0 126 043-A1, disclose
saccharides that can be used in the treatment,
prophylaxis or diagnosis of bacterial infections
caused by *Streptococci pneumoniae*. One class of
useful saccharides is derived from the disaccharide
GlcNAcβ1 3Gal. Andersson et al. also reported
neolactotetraosylceramide to be useful, which is
Galβ1 4GlcNAcβ1 3Galβ1 4Glc-Cer.

McKenney et al. (1999) *Science*, **284**:1523-
1527, disclose a polysaccharide, poly-N-succinyl
β1 6GlcN (PNSG) that provides protection from
Staphylococcus aureus. *S. aureus* is a common cause
of community-acquired infections, including
endocarditis, osetemylitis, septic arthritis,
pneumonia, and abscesses.

European Patent No. 0 157 899-B1 discloses
the isolation of pneumococcal polysaccharides that
are useful in the present invention. The following
table lists the pneumococcal culture types that
produce capsular polysaccharides useful as haptens in
the present invention.

Polysaccharide Hapten Sources

Danish Type Nomenclature	U.S. Nomenclature	1978 ATCC Catalogue Number
1	1	6301
2	2	6302
3	3	6303
4	4	6304
5	5	
6A	6	6306
6B	26	6326
7F	51	10351
8	8	6308
9N	9	6309
9V	68	
10A	34	
11A	43	
12F	12	6312
14	14	6314
15B	54	
17F	17	
18C	56	10356
19A	57	
19F	19	6319
20	20	6320
22F	22	
23F	23	6323
25	25	6325
33F	70	

Moraxella (Branhamella) catarrhalis is a reported cause of otitis media and sinusitis in children and lower respiratory tract infections in adults. The lipid A portion of the lipooligo-saccharide surface antigen (LOS) of the bacterium is cleaved at the 3-deoxy-D-manno-octulosonic acid-

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glucosamine linkage. The cleavage product is treated with mild-alkali to remove ester-linked fatty acids, while preserving amide-linked fatty acids to yield detoxified lipopolysaccharide (dLOS) from *M. catarrhalis*. The dLOS is not immunogenic until it is attached to a protein carrier. Xin-Xing Gu et al. (1998) *Infect. Immun.*, **66(5)**:1891-1897.

Group B streptococci (GBS) is a cause of sepsis, meningitis, and related neurologic disorders in humans. The Capsular polysaccharide-specific antibodies are known to protect human infants from infection. Jennings et al., U.S. Patent No. 5,795,580. The repeating unit of the GBS capsular polysaccharide type II is: 4)-β-D-GlcpNAc-(1 3)-[β-D-Galp(1 6)]-β-D-Galp(1 4)-β-D-Glcp-(1 3)-β-D-Glcp-(1 2)-[α-D-NeupNAc(2 3)]-β-D-Galp-(1 , where the bracketed portion is a branch connected to the immediately following unbracketed subunit. The repeating unit of GBS capsular polysaccharide type V is: 4)-[α-D-NeupNAc-(2 3)-β-D-Galp-(1 4)-β-D-GlcpNAc-(1 6)]-α-D-Glcp-(1 4)-[β-D-Glcp-(1 3)]-β-D-Galp-(1 4)-β-D-Glcp-(1 .

European patent application No. EU-0 641 568-A1, Brade, discloses the method of obtaining ladder-like banding pattern antigen from *Chlamydia trachomatis*, *pneumoniae* and *psittaci*.

Slovin et al., (1999) *Proc. Natl. Acad. Sci., U.S.A.*, **96(10)**:5710-5715 report use of a synthetic oligosaccharide, globo H, linked to KLH as a carrier in the preparation of a vaccine used against prostate cancer. Similarly, Helling et al., (July 1995) *Cancer Res.*, 55:2783-2788 report the use of KLH-linked G_{M2} in a vaccine for treating patients

with melanoma. The latter vaccine was prepared by ozone cleavage of the ceramide double bond of G_{M2}, introduction of an aldehyde group and reductive alkylation onto KLH. A similar procedure can be utilized with a contemplated chimer particle.

Oligosaccharidal portions of sphingolipids such as globosides and gangliosides that are present on the surface of other tumor cells as well as normal cells such as melanoma, neuroblastoma and healthy brain cells can similarly be used herein as a hapten. The oligosaccharide portion of the globoside globo H has the structure Fucα-(1 2)-Galβ(1 3)-GalNAcβ-(1 3)-Galα-(1 4)-Galβ-(1 4)Glc, whereas the saccharide protions of gangliosides G_{M2}, G_{M1} and G_{D1a} have the following structures: GalNAcβ-(1 4)-[NeuAcα-(2 3)]-Galβ-(1 4)-Glc; Galβ-(1 3)-GalNAcβ-(1 4)-[NeuAcα-(2 3)]-Galβ-(1 4)-Glc; and NeuAc-(2 3)-Galβ-(1 3)-GalNAcβ-(1 4)-[NeuAcα-(2 3)]-Galβ-(1 4)-Glc, respectively.

U.S. Patent No. 4,356,170 discloses the preparation of useful polysaccharides that are reduced and then oxidized to form compounds having terminal aldehyde groups that can be reductively aminated onto free amine groups of carrier proteins such as tetanus toxoid and diphtheria toxoid with or without significant cross-linking. Exemplary useful bacterial polysaccharides include β-hemolytic streptococci, *Haemophilus influenza*, meningococci, pneumococci and *E. coli*. Rather than reductively aminating the particles, a linker arm such as that provided by an ε-amino C_2-C_8 alkylcarboxylic acid can be reductively aminated on to the polysaccharide,

followed by linkage to the particles using a water-soluble carbodiimide.

Inocula and Vaccines

In yet another embodiment of the invention, a HBc chimer particle or HBc chimer particle conjugate with a hapten is used as the immunogen of an inoculum that induces a B cell or T cell response (stimulation) in an inoculated host animal such as production of antibodies that immunoreact with the heterologous epitope or hapten or T cell activation, or as a vaccine to provide protection against the pathogen, protein or other entity from which the heterologous epitope or the hapten is derived.

T cell activation can be measured by a variety of techniques. In usual practice, a host animal is inoculated with a contemplated HBc chimer particle vaccine or inoculum, and peripheral mononuclear blood cells (PMBC) are thereafter collected. Those PMBC are then cultured *in vitro* in the presence of the T cell immunogen for a period of about three to five days. The cultured PMBC are then assayed for proliferation or secretion of a cytokine such as IL-2, GM-CSF of IFN-γ. Assays for T cell activation are well known in the art. See, for example, U. S. Patent No. 5,478,726 and the art cited therein.

Using antibody formation as exemplary, a contemplated inoculum or vaccine comprises an immunogenic effective amount of HBc chimer particles or HBc chimer particle conjugates that are dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered to a host animal in need of immunization

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or in which antibodies are desired to be induced such as a mammal (e.g., a mouse, dog, goat, sheep, horse, bovine, monkey, ape, or human) or bird (e.g., a chicken, turkey, duck or goose), an inoculum induces antibodies that immunoreact with the conjugated (pendently-linked) hapten. Those antibodies also preferably bind to the protein or saccharide of the B cell immunogen.

A vaccine is a type of inoculum in which the heterologous B cell epitope or conjugated hapten corresponds to a portion of a protein or saccharidal structure that is related to a disease state, as is an exemplary malarial B cell sequence related to a malarial pathogen. The vaccine-induced antibodies not only immunoreact with the epitope or hapten or activated T cells respond to that heterologous epitope or hapten, but also immunoreact with the pathogen or diseased cell *in vivo*, and provide protection from that disease state.

The amount of recombinant HBc chimer immunogen utilized in each immunization is referred to as an immunogenic effective amount and can vary widely, depending *inter alia*, upon the recombinant HBc chimer immunogen, mammal immunized, and the presence of an adjuvant in the vaccine, as discussed below. Immunogenic effective amounts for a vaccine and an inoculum provide the protection or antibody activity, respectively, discussed hereinbefore.

Vaccines or inocula typically contain a recombinant HBc chimer immunogen concentration of about 1 microgram to about 1 milligram per inoculation (unit dose), and preferably about 10 micrograms to about 50 micrograms per unit dose. The term "unit dose" as it pertains to a vaccine or inoculum of the present invention

refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to individually or collectively produce the desired immunogenic effect in association with the required diluent; i.e., carrier, or vehicle.

Vaccines or inocula are typically prepared from a recovered recombinant HBc chimer immunogen by dispersing the immunogen, preferably in particulate form, in a physiologically tolerable (acceptable) diluent vehicle such as water, saline phosphate-buffered saline (PBS), acetate-buffered saline (ABS), Ringer's solution or the like to form an aqueous composition. The diluent vehicle can also include oleaginous materials such as peanut oil, squalane or squalene as is discussed hereinafter.

The preparation of inocula and vaccines that contain proteinaceous materials as active ingredients is also well understood in the art. Typically, such inocula or vaccines are prepared as parenterals, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified, which is particularly preferred.

The immunogenic active ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, an inoculum or vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying

agents, pH buffering agents that enhance the immunogenic effectiveness of the composition.

A contemplated vaccine or inoculum advantageously also includes an adjuvant. Suitable adjuvants for vaccines and inocula of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against B cell epitopes of the chimer, as well as adjuvants capable of enhancing cell mediated responses towards T cell epitopes contained in the chimer. Adjuvants are well known in the art (see, for example, Vaccine Design - The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X).

Exemplary adjuvants include complete Freund's adjuvant (CFA) that is not used in humans, incomplete Freund's adjuvant (IFA), squalene, squalane and alum [e.g., Alhydrogel™ (Superfos, Denmark)], which are materials well known in the art, and are available commercially from several sources.

Preferred adjuvants for use with immunogens of the present invention include aluminum or calcium salts (for example hydroxide or phosphate salts). A particularly preferred adjuvant for use herein is an aluminum hydroxide gel such as Alhydrogel™. For aluminum hydroxide gels, the chimer protein is admixed with the adjuvant so that between 50 to 800 micrograms of aluminum are present per dose, and preferably between 400 and 600 micrograms are present.

Another particularly preferred adjuvant for use with an immunogen of the present invention is an emulsion. A contemplated emulsion can be an oil-in-

water emulsion or a water-in-oil emulsions. In addition to the immunogenic chimer protein, such emulsions comprise an oil phase of squalene, squalane, peanut oil or the like as are well-known, and a dispersing agent. Non-ionic dispersing agents are preferred and such materials include mono- and di-C_{12}-C_{24}-fatty acid esters of sorbitan and mannide such as sorbitan mono-stearate, sorbitan mono-oleate and mannide mono-oleate. An immunogen-containing emulsion is administered as an emulsion.

Preferably, such emulsions are water-in-oil emulsions that comprise squalene and mannide mono-oleate (Arlacel™ A), optionally with squalane, emulsified with the chimer protein in an aqueous phase. Well-known examples of such emulsions include Montanide™ ISA-720, and Montanide™ ISA 703 (Seppic, Castres, France), each of which is understood to contain both squalene and squalane, with squalene predominating in each, but to a lesser extent in Montanide™ ISA 703. Most preferably, Montanide™ ISA-720 is used, and a ratio of oil-to-water of 7:3 (w/w) is used. Other preferred oil-in-water emulsion adjuvants include those disclosed in WO 95/17210 and EP 0 399 843.

The use of small molecule adjuvants is also contemplated herein. One type of small molecule adjuvant useful herein is a 7-substituted-8-oxo- or 8-sulfo-guanosine derivative described in U.S. Patents No. 4,539,205, No. 4,643,992, No. 5,011,828 and No. 5,093,318, whose disclosures are incorporated by reference. Of these materials, 7-allyl-8-oxoguanosine (loxoribine) is particularly preferred. That molecule has been shown to be particularly

effective in inducing an antigen-(immunogen-)specific response.

Still further useful adjuvants include monophosphoryl lipid A (MPL) available from Corixa Corp. (see, U.S. Patent No. 4,987,237), CPG available from Coley Pharmaceutical Group, QS21 available from Aquila Biopharmaceuticals, Inc., SBAS2 available from SKB, the so-called muramyl dipeptide analogues described in U.S. Patent No. 4,767,842, and MF59 available from Chiron Corp. (see, U.S. Patents No. 5,709,879 and No. 6,086,901).

More particularly, immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree *Quillaja Saponaria Molina* (e.g. Quil™ A) are also useful. Derivatives of Quil™ A, for example QS21 (an HPLC purified fraction derivative of Quil™ A), and the method of its production is disclosed in U.S. Patent No.5,057,540. In addition to QS21 (known as QA21), other fractions such as QA17 are also disclosed.

3-De-O-acylated monophosphoryl lipid A is a well-known adjuvant manufactured by Ribi Immunochem, Hamilton, Montana. The adjuvant contains three components extracted from bacteria, monophosphoryl lipid (MPL) A, trehalose dimycolate (TDM) and cell wall skeleton (CWS) (MPL+TDM+CWS) in a 2% squalene/Tween® 80 emulsion. This adjuvant can be prepared by the methods taught in GB 2122204B. A preferred form of 3-de-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2 μm in diameter (EP 0 689 454 B1).

The muramyl dipeptide adjuvants include N-acetyl-muramyl-L-threonyl-D-isoglutamine(thur-MDP),

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N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP
11637, referred to as nor-MDP), and N-acetylmuramyl-
L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-
dipalmityol-sn-glycero-3-hydroxyphosphoryloxy)-
ethylamin (CGP) 1983A, referred to as MTP-PE).

Preferred adjuvant mixtures include
combinations of 3D-MPL and QS21 (EP 0 671 948 B1),
oil-in-water emulsions comprising 3D-MPL and QS21 (WO
95/17210, PCT/EP98/05714), 3D-MPL formulated with
other carriers (EP 0 689 454 B1), QS21 formulated in
cholesterol-containing liposomes (WO 96/33739), or
immunostimulatory oligonucleotides (WO 96/02555).
Alternative adjuvants include those described in WO
99/52549 and non-particulate suspensions of
polyoxyethylene ether (UK Patent Application No.
9807805.8).

Adjuvants are utilized in an adjuvant
amount, which can vary with the adjuvant, mammal and
recombinant HBc chimer immunogen. Typical amounts
can vary from about 1 μg to about 1 mg per
immunization. Those skilled in the art know that
appropriate concentrations or amounts can be readily
determined.

Inocula and vaccines are conventionally
administered parenterally, by injection, for example,
either subcutaneously or intramuscularly. Additional
formulations that are suitable for other modes of
administration include suppositories and, in some
cases, oral formulation. The use of a nasal spray
for inoculation is also contemplated as discussed in
Neirynck et al. (Oct. 1999) *Nature Med.*, **5(10)**:1157-
1163. For suppositories, traditional binders and
carriers can include, for example, polyalkalene
glycols or triglycerides; such suppositories may be

formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like.

An inoculum or vaccine composition takes the form of a solution, suspension, tablet, pill, capsule, sustained release formulation or powder, and contains an immunogenic effective amount of HBc chimer or HBc chimer conjugate, preferably as particles, as active ingredient. In a typical composition, an immunogenic effective amount of preferred HBc chimer or HBc chimer conjugate particles is about 1 μg to about 1 mg of active ingredient per dose, and more preferably about 5 μg to about 50 μg per dose, as noted before.

A vaccine is typically formulated for parenteral administration. Exemplary immunizations are carried out sub-cutaneously (SC) intra-muscularly (IM), intravenusly (IV), intraperitoneally (IP) or intra-dermally (ID). However, oral and nasal routes of vaccination are also contemplated.

The HBc chimer particles and HBc chimer particle conjugates can be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein or hapten) and are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived form inorganic

bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

In yet another embodiment, a vaccine or inoculum is contemplated in which a gene encoding a contemplated HBc chimer is transfected into suitably attenuated enteric bacteria such as *S. typhi, S. typhimurium, S. typhimurium-E. coli* hybrids or *E. coli*. Exemplary attenuated or avirulent *S. typhi* and *S. typhimurium* and *S. typhimurium-E. coli* hybrids are discussed in the citations provided before. These vaccines and inocula are particularly contemplated for use against diseases that infect or are transmitted via mucosa of the nose, the gut and reproductive tract such as influenza, yeasts such as *Aspergiullus* and *Candida*, viruses such as polio, moot-and-mouth disease, hepatitis A, and bacteria such as *Cholera*, *Salmonella* and *E. coli* and where a mucosal IgA response is desired in addition to or instead of an IgG systemic response.

The enteric bacteria can be freeze dried, mixed with dry pharmaceutically acceptable diluents, made into tablets or capsules for ingestion and administered to or taken by the host animal as are usual solid phase medications. In addition, aqueous preparations of these bacterial vaccines are adapted for use in mucosal immunization as by oral, nasal, rectal or vaginal administration.

Oral immunization using plant matter containing contemplated chimeric molecule particles can be achieved by simple ingestion of the transgenic plant tissue such as a root like a carrot or seed

such as rice or corn. In this case, the water of the
mouth or gastrointestinal tract provides the usually
used aqueous medium used for immunization and the
surrounding plant tissue provides the
pharmaceutically acceptable diluent.

The inocula or vaccines are administered in
a manner compatible with the dosage formulation, and
in such amount as are therapeutically effective and
immunogenic. The quantity to be administered depends
on the subject to be treated, capacity of the
subject's immune system to synthesize antibodies, and
degree of protection desired. Precise amounts of
active ingredient required to be administered depend
on the judgment of the practitioner and are peculiar
to each individual. However, suitable dosage ranges
are of the order of tens of micrograms active
ingredient per individual. Suitable regimes for
initial administration and booster shots are also
variable, but are typified by an initial
administration followed in intervals (weeks or
months) by a subsequent injection or other
administration.

Once immunized, the mammal is maintained
for a period of time sufficient for the recombinant
HBc chimer immunogen to induce the production of a
sufficient titer of antibodies that bind to an
antigen of interest such as a sporozoite for a
malarial vaccine. The maintenance time for the
production of illustrative anti-sporozoite antibodies
typically lasts for a period of about three to about
twelve weeks, and can include a booster, second
immunizing administration of the vaccine. A third
immunization is also contemplated, if desired, at a
time 24 weeks to five years after the first

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immunization. It is particularly contemplated that once a protective level titer of antibodies is attained, the vaccinated mammal is preferably maintained at or near that antibody titer by periodic booster immunizations administered at intervals of about 1 to about 5 years.

The production of anti-sporozoite or other antibodies is readily ascertained by obtaining a plasma or serum sample from the immunized mammal and assaying the antibodies therein for their ability to bind to an approriate antigen such as a synthetic circumsporozoite immunodominant antigen [e.g. the *P. falciparum* CS protein peptide (NANP)$_5$ used herein] in an ELISA assay as described hereinafter or by another immunoassay such as a Western blot as is well known in the art.

It is noted that the induced antibodies such as anti-CS antibodies or anti-influenza antibodies can be isolated from the blood of an inoculated host mammal using well known techniques, and then reconstituted into a second vaccine for passive immunization as is also well known. Similar techniques are used for gamma-globulin immunizations of humans. For example, antiserum from one or a number of immunized hosts can be precipitated in aqueous ammonium sulfate (typically at 40-50 percent of saturation), and the precipitated antibodies purified chromatographically as by use of affinity chromatography in which (NANP)$_5$ or an influenza M2 polypeptide is utilized as the antigen immobilized on the chromatographic column. Thus, for example, an inoculum can be used in a horse or sheep to induce antibody production against a malarial species for

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use in a passive immunization in yet another animal such as humans.

Another embodiment of the invention is a process for inducing antibodies, activated T cells or both in an animal host comprising the steps of inoculating said animal host with an inoculum. The inoculum used in the process comprises an immunogenic amount of a before-described HBc chimer particle or HBc chimer particle conjugate dissolved or dispersed in a pharmaceutically acceptable diluent. The animal host is maintained for a time sufficient for antibodies or activated T cells to be induced, as can be assayed by well-known techniques, which typically requires a time period of weeks to months, as is again well-known. A plurality of such immunizations is contemplated during this maintenance period.

The invention is illustrated by the following non-limiting examples.

Example 1: B Cell Epitope-Containing
            Chimer Preparation

            A.   Preparation of plasmid vector pKK223-
                  3N, a modified form of pKK223-3
            Plasmid vector pKK223-3 (Pharmacia) was modified by the establishment of a unique NcoI restriction site to enable insertion of HBc genes as NcoI-HindIII restriction fragments and subsequent expression in *E.coli* host cells. To modify the pKK223-3 plasmid vector, a new SphI-HindIII fragment was prepared using the PCR primers pKK223-3/433-452-F and pKK223-NcoI-mod-R, and pKK223-3 as the template. This PCR fragment was cut with the restriction enzymes SphI and HindIII to provide a 467 bp fragment

that was then ligated with a 4106 bp fragment of the
pKK223-3 vector, to effectively replace the original
480 bp SphI-HindIII fragment. The resultant plasmid
(pKK223-3N) is therefore 13 bp shorter than the
parent plasmid and contains modified nucleotide
sequence upstream of the introduced NcoI site (see
Fig. 1 in which the dashes indicate the absent
bases). The final plasmid, pKK223-3N, has a size of
4573 bp. Restriction sites in plasmid pKK223-3N are
indicated in Fig. 1, and the nucleotide changes made
to pKK223-3 to form plasmid pKK223-3N are indicated
by an underline as shown below.

pKK223-3/433-452-F   GGTGCATGCAAGGAGATG   SEQ ID NO:178

pKK223-NcoI-mod-R
GCGAAGCTTCGGATCccatggTTTTTTCCTCCTTATGTGAAATTGTTATCCG-
        CTC   SEQ ID NO:179

      B.   Preparation of V1
          and V2 Cloning Vectors
      Modified HBc149 genes, able to accept the
directional insertion of synthetic dsDNA fragments
into the immunodominant loop region, were constructed
using PCR. [The plasmid accepting inserts between
amino acids E77 and D78 was named V1, whereas the
plasmid accepting inserts between D78 and P79 was
named V2.] The HBc149 gene was amplified in two
halves using two PCR primer pairs, one of which
amplifies the amino terminus, the other amplifies the
carboxyl terminus. For V1, the products of the PCR
reactions (N- and C-terminus) are both 246 bp
fragments; for V2, the products are a 249 bp (N-
terminus) and a 243 bp fragment (C-terminus).

The N-terminal fragments prepared were digested with NcoI and EcoRI, and the C-terminal fragments were digested with EcoRI and HindIII. The V1 and V2 fragments pairs were then ligated together at the common EcoRI overhangs. The resultant NcoI-HindIII fragments were then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

To insert B cell epitopes into the V1 and V2 plasmids, the plasmids were digested with EcoRI and SacI restriction enzymes. Synthetic dsDNA fragments containing 5' EcoRI and 3' SacI overhangs were then inserted. In both cases, V1 and V2, glycine-isoleucine (EcoRI) and glutamic acid-leucine (SacI) amino acid pairs, coded for by the restriction sites, flank the inserted B cell epitopes. The inserted restriction sites are underlined in the primers below.

V1

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA   SEQ ID NO:180

5'-GGTGGCTTACCAGAGGCCGGAGGC

          AATGGATCATTCGAACCG   SEQ ID NO:290

HBc-E77/EcoRI-R

5'-GCGGAATTCCTTCCAAATTAACACCCACC   SEQ ID NO:181

HBc-D78/EcoRI-SacI-F

5'-CGCGAATTCAAAAAGAGCTCGATCCAGCGTCTAGAGAC

                                     SEQ ID NO:182

HBc149/HindIII-R

5'-CGC<u>AAGCTT</u>AAACAACAGTAGTCTCCGGAAG   SEQ ID NO:183

V2

HBc149/NcoI-F

5'-TTGGG<u>CCATGG</u>ACATCGACCCTTA   SEQ ID NO:180

HBc-D78/EcoRI-R

5'-GCG<u>GAATTC</u>CATCTTCCAAATTAACACCCAC   SEQ ID NO:184

HBc-P79/EcoRI-SacI-F

5'-CGC<u>GAATTC</u>AAAAA<u>GAGCTC</u>CCAGCGTCTAGAGACCTAG

                  SEQ ID NO:185

HBc149/HindIII-R

5'-CGC<u>AAGCTT</u>AAACAACAGTAGTCTCCGGAAG   SEQ ID NO:183

Vectors to Express Chimer Particles Containing an N-Terminal Cysteine and the CS-Repeat Epitopes from *P.falciparum* in the Immunodominant Loop

      Two expression vectors [V2.Pf1(N-MGCELDP) and V2.Pf1(N-MGCDIDP)] are prepared to determine the ability of N-terminal cysteine residues to stabilize chimer particles. To make the vector V2.Pf1(N-MGCELDP), the oligonucleotides HBc(MGCELDP)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 528 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which had been cleaved with the same two enzymes.

To make the vector V2.Pf1(N-MGCDIDP) the oligonucleotides HBc(MGCDIDP)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 528 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which has been cleaved with the same two enzymes.

HBc(MGCELDP)-NcoI-F

    M   G   C   E   L   D   P   Y   K   E   F   G   SEQ ID NO:186
5'-GCGCCATGGGGTGTGAGCTCGACCCTTATAAAGAATTTGG   SEQ ID NO:187

HBc(MGCDIDP)-NcoI-F

    M   G   C   D   I   D   P   Y   K   E   F   G   SEQ ID NO:188
5'-GCGCCATGGGGTGTGACATCGACCCTTATAAAGAATTTGG   SEQ ID NO:189

C.   Preparation of V7 Cloning Vector

To enable the fusion of T cell epitopes to the C terminus of a HBc chimer, a new vector, V7, was constructed. Unique EcoRI and SacI restriction sites were inserted between valine-149 and the HindIII site to facilitate directional insertion of synthetic dsDNAs into EcoRI-HindIII (or EcoRI-SacI) restriction sites. The pair of PCR primers below was used to amplify the HBc 149 gene with a NcoI restriction site at the amino-terminus and EcoRI, SacI and HindIII sites at the carboxyl-terminus. The product of the PCR reaction (479 bp) was digested with NcoI/HindIII and cloned into pKK223-3N to form V7.

To insert T cell epitopes, the plasmid (V7) was digested EcoRI/HindIII (or EcoRI-SacI) and synthetic dsDNA fragments having EcoRI/HindIII (or

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EcoRI/SacI) overhangs, were ligated into V7. For all
V7 constructs, the final amino acid of native HBc
(valine-149) and the first amino acid of the inserted
T cell epitope are separated by a glycine-isoleucine
dipeptide sequence coded for by the nucleotides that
form the EcoRI restriction site. For epitopes
inserted at EcoRI/SacI, there are additional glutamic
acid-leucine residues after the T cell epitope, prior
to the termination codon, contributed by the SacI
site. Restriction sites are again underlined in the
primers shown.

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA   SEQ ID NO:180

HBc149/SacI-EcoRI-H3-R

5'-CGCAAGCTTAGAGCTCTTGAATTCCAACAACAGTAGTCTCCG

                                          SEQ ID NO:190

            D.   Preparation of V12

            Expression Constructs

        V12 vectors, which contain B cell epitopes
between amino acids 78 and 79, as well as T cell
epitopes downstream of valine-149, are constructed
from V2 and V7 vectors. The carboxyl terminus of a
V7 vector containing a T cell epitope inserted at
EcoRI/HindIII is amplified using two PCR primers
(HBc-P79/SacI-F and pKK223-2/4515-32R) to provide a
dsDNA fragment corresponding to amino acids 79-149
plus the T cell epitope, flanked with SacI and
HindIII restriction sites.

        The PCR products are cut with SacI and
HindIII and then cloned into the desired V2 vector
prepared by cutting with the same two enzymes. The

PCR primers are amenable for the amplification of the carboxyl terminus of all V7 genes, irrespective of the T cell epitope present after amino acid 149 of the HBc gene.

One exception to the generality of this approach was in the preparation of the V12 constructs containing the Pf-CS(C17A) mutation, which were prepared from existing V12 constructs. In this case, V12 constructs were amplified with HBc149/NcoI-F (SEQ ID NO:180) and the mis-match reverse PCR primer (SEQ ID NO:292), which facilitated the C17A mutation. The resultant PCR product was digested with NcoI and HindIII and cloned back into pKK223-3N (previously cut with the same enzymes). Restriction sites are underlined.

HBc-P79/SacI-F   5'-CGCGAGCTCCCAGCGTCTAGAGACCTAG

                                      SEQ ID NO:191

pKK223-2/4515-32R   5'-GTATCAGGCTGAAAATC

                                      SEQ ID NO:192

    E.   *P.falciparum* CS-repeat B cell
         Epitopes Inserted into V2
    For V2 and V7 constructs, synthetic dsDNA fragments coding for the B (V2) or T cell epitope (V7) of interest are inserted into EcoRI/SacI restriction sites. Synthetic dsDNA fragments, encoding B and T cell epitopes of interest, are prepared by mixing complementary single stranded DNA oligonucleotides at equimolar concentrations, heating to 95°C for 5 minutes, and then cooling to room temperature at a rate of -1 °C per minute. This annealing reaction is performed in TE buffer. The

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double-stranded DNAs are shown below with the encoded
epitope sequence shown above. The pound symbol, #,
is used in some of the amino acid residue sequences
that follow to indicate the presence of a stop codon.

Pf1

    I   N   A   N   P   N   A   N   P   N   A   N   P   N   A
AATTAACGCTAATCCGAACGCTAATCCGAACGCTAATCCGAACGCTA
    TTGCGATTAGGCTTGCGATTAGGCTTGCGATTAGGCTTGCGAT

            N   P   E   L   SEQ ID NO:193
            ATCCGGAGCT   SEQ ID NO:194
            TAGGCC   SEQ ID NO:195

Pf3

    I   N   A   N   P   N   V   D   P   N   A   N   P   N   A   N   P
AATTAACGCTAATCCGAACGTTGACCCGAACGCTAATCCGAACGCTAATCCGA
    TTGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCTTGCGATTAGGCT

N   A   N   P   N   V   D   P   N   A   N   P   E   L SEQ ID NO:196
ACGCTAATCCGAACGTTGACCCGAACGCTAATCCGGAGCT SEQ ID NO:197
TGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCCTCGAGG
                                        SEQ ID NO:198

Pf3.1

    I   N   A   N   P   N   V   D   P   N   A   N   P   N   A   N   P
AATTAACGCGAATCCGAACGTGGATCCGAATGCCAACCCTAACGCCAACCC
TTGCGCTTAGGCTTGCACCTAGGCTTACGGTTGGGATTGCGGTTGGG

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```
        N   A   N   P   E   L           SEQ ID NO:199
      AAATGCGAACCCAGAGCT                SEQ ID NO:200
      TTTACGCTTGGGTC                    SEQ ID NO:201
```

Pf3.2

```
  I   N   A   N   P   N   A   N   P   N   A   N   P   N   V   D   P
AATTAACGCGAATCCGAATGCCAACCCTAACGCCAACCCAAACGTGGATCCGA
    TTGCGCTTAGGCTTACGGTTGGGATTGCGGTTGGGTTTGCACCTAGGCT
```

```
        N   A   N   P   E   L           SEQ ID NO:202
      ATGCGAACCCAGAGCT                  SEQ ID NO:203
      TACGCTTGGGTC                      SEQ ID NO:204
```

Pf3.3

```
  I   N   A   N   P   N   V   D   P   N   A   N   P   N   A   N   P
AATTAACGCGAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAA
    TTGCGCTTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTT
```

```
N   A   N   P   N   V   D   P   N   A   N   P   E   L   SEQ ID NO:205
ACGCCAACCCGAATGTTGACCCCAATGCCAATCCGGAGCT              SEQ ID NO:206
TGCGGTTGGGCTTACAACTGGGGTTACGGTTAGGCC                  SEQ ID NO:207
```

Pf3.4

```
  I   N   P   N   V   D   P   N   A   N   P   N   A   N   P   N   A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
    TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT
```

Pf3.5

I N P N V D P N A N P N A N P N A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
    TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT

Pf3.6

I N P N V D P N A N P N A N P N A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
    TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT

Pf3.7

I N V D P N A N P N A N P N A N P
AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
    TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT

```
          N   V   E   L            SEQ ID NO:217

          ATGTTGAGCT               SEQ ID NO:218

          TACAAC                   SEQ ID NO:219
```

Pf3.8

```
   I   N   V   D   P   N   A   N   P   N   A   N   P   N   A   N   P
AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
   TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT
```

```
          N   V   D   P   E   L            SEQ ID NO:220

          ATGTTGACCCTGAGCT                 SEQ ID NO:221

          TACAACTGGGAC                     SEQ ID NO:222
```

Pf3.9

```
   I   N·  V   D   P   N   A   N   P   N   A   N   P   N   A   N   P
AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
   TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT
```

```
          N   V   D   P   N   A   E   L        SEQ ID NO:223

          ATGTTGACCCTAATGCTGAGCT               SEQ ID NO:224

          TACAACTGGGATTACGAC                   SEQ ID NO:225
```

Pf3.10

```
   I   D   P   N   A   N   P   N   A   N   P   N   A   N   P
AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACC
   CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGG
```

```
          N     V     E     L          SEQ ID NO:226

          CGAATGTTGAGCT                 SEQ ID NO:227
```

```
             GCTTACAAC              SEQ ID NO:228
```

Pf3.11

```
   I  D  P  N  A  N  P  N  A  N  P  N  A  N  P  N  V
AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
     CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCTTACAAC
```

```
             D  P  E  L              SEQ ID NO:229
             ACCCTGAGCT              SEQ ID NO:230
             TGGGAC                  SEQ ID NO:231
```

Pf3.12

```
   I  D  P  N  A  N  P  N  A  N  P  N  A  N  P  N  V
AATTGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
     CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCTTACAAC
```

```
             D  P  N  A  E  L        SEQ ID NO:232
             ACCCTAATGCCGAGCT        SEQ ID NO:233
             TGGGATTACGGC            SEQ ID NO:234
```

F.   *P.falciparum* universal T cell epitope

Pf-UTC (PF/CS326-345)

```
   I  E  Y  L  N  K  I  Q  N  S  L  S  T  E  W  S  P
AATTGAATATCTGAACAAAATCCAGAACTCTCTGTCCACCGAATGGTCTCCGT
     CTTATAGACTTGTTTTAGGTCTTGAGAGACAGGTGGCTTACCAGAGGCA
```

```
C   S   V   T   #   #                SEQ ID NO:235

GCTCCGTTACCTAGTA                     SEQ ID NO:236

CGAGGCAATGGATCATTCGA                 SEQ ID NO:237
```

P.vivax CS-repeat B cell epitopes

Pv-T1A

```
I   P   A   G   D   R   A   D   G   Q   P   A   G   D   R   A   A
AATTCCGGCTGGTGACCGTGCAGATGGCCAGCCAGCGGGTGACCGCGCTGCAG
      GGCCGACCACTGGCACGTCTACCGGTCGGTCGCCCACTGGCGCGACGTC
```

```
G   Q   P   A   G   E   L                SEQ ID NO:238

GCCAGCCGGCTGGCGAGCT                      SEQ ID NO:239

CGGTCGGCCGACCGC                          SEQ ID NO:240
```

Pv-T1B

```
I   D   R   A   A   G   Q   P   A   G   D   R   A   D   G   Q   P
AATTGACAGAGCAGCCGGACAACCAGCAGGCGATCGAGCAGACGGACAGCCCG
      CTGTCTCGTCGGCCTGTTGGTCGTCCGCTAGCTCGTCTGCCTGTCGGGC
```

```
A   G   E   L                            SEQ ID NO:241

CAGGGGAGCT                               SEQ ID NO:242

GTCCCC                                   SEQ ID NO:243
```

Pv-T2A

```
I   A   N   G   A   G   N   Q   P   G   A   N   G   A   G   D   Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGGGGCAAACGGCGCGGGTGATCAAC
      CGCTTGCCGCGGCCATTAGTCGGCCCCCGTTTGCCGCGCCCACTAGTTG
```

```
              P   G   E   L                     SEQ  ID  NO:244

              CAGGGGAGCT                         SEQ  ID  NO:245

              GTCCCC                             SEQ  ID  NO:246
```

Pv-T2B

```
      I  A  N  G  A  D  N  Q  P  G  A  N  G  A  D  D  Q
AATTGCGAACGGCGCCGATAATCAGCCGGGTGCAAACGGGGCGGATGACCAAC
      CGCTTGCCGCGGCTATTAGTCGGCCCACGTTTGCCCCGCCTACTGGTTG
```

```
              P   G   E   L                     SEQ  ID  NO:247

              CAGGCGAGCT                         SEQ  ID  NO:248

              GTCCGC                             SEQ  ID  NO:249
```

Pv-T2C

```
      I  A  N  G  A  G  N  Q  P  G  A  N  G  A  G  D  Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGAGCAAACGGCGCGGGGGATCAAC
      CGCTTGCCGCGGCCATTAGTCGGCCCTCGTTTGCCGCGCCCCCTAGTTG
```

```
P  G  A  N  G  A  D  N  Q  P  G  A  N  G  A  D  D
CAGGCGCCAATGGTGCAGACAACCAGCCTGGGGCGAATGGAGCCGATGACC
GTCCGCGGTTACCACGTCTGTTGGTCGGACCCCGCTTACCTCGGCTACTGG
```

```
              Q   P   G   E   L                 SEQ  ID  NO:250

              AACCCGGCGAGCT                      SEQ  ID  NO:251

              TTGGGCCGC                          SEQ  ID  NO:252
```

PV-T3

```
I  A  P  G  A  N  Q  E  G  G  A  A  A  P  G  A  N
AATTGCGCCGGGCGCCAACCAGGAAGGTGGGGCTGCAGCGCCAGGAGCCAATC
```

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CGCGGCCCGCGGTTGGTCCTTCCACCCCGACGTCGCGGTCCTCGGTTAG

Q   E   G   G   A   A   E L   SEQ ID NO:253

AAGAAGGCGGTGCAGCGGAGCT   SEQ ID NO:254

TTCTTCCGCCACGTCGCC   SEQ ID NO:255

Example 2: Assay Procedures

    A. Antigenicity

    1. Particle ELISA

Purified particles were diluted to a concentration of 10 μg/mL in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 μL/well). The ELISA strips are incubated at room temperature overnight (about 18 hours). Next morning the wells are washed with ELISA wash buffer [phosphate buffered saline (PBS), pH 7.4, 0.05% Tween®-20] and blocked with 3% BSA in PBS for 1 hour (75 μL/well). ELISA strips are stored, dry, at -20°C until needed.

To determine the antigenicity of particles, antisera are diluted using 1% BSA in PBS and 50 μL/well added to antigen-coated ELISA wells. Sera are incubated for 1 hour, washed with ELISA wash buffer and probed using an anti-mouse(IgG)-HRP (The Binding Site, San Diego, CA; HRP = horseradish peroxidase) conjugate (50 μL/well) or other appropriate antibody for 30 minutes. After washing with ELISA wash buffer the reaction is visualized by the addition of TM blue substrate (50 μL/well). After 10 minutes, the reaction is stopped by the addition of 1N H_2SO_4 (100 μL/well) and is read on an ELISA plate reader set at 450 nm.

2. Synthetic Peptide ELISA

A 20 amino acid residue synthetic peptide (NANP)$_5$ is diluted to a concentration of 2 μg/mL in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 μL/well). Peptides are dried onto the wells by incubating overnight (about 18 hours), in a hood with the exhaust on. Next morning, the wells are washed with ELISA wash buffer (phosphate buffered saline, pH 7.4, 0.05% Tween®-20) and blocked with 3% BSA in PBS (75 μL/well) for 1 hour. ELISA strips are stored, dry, at -20°C until needed.

To determine antibody antigenicity of particles, antisera (monoclonal or polyclonal) are diluted using 1% BSA in PBS, and 50 μL/well are added to antigen-coated ELISA wells. Sera are incubated for 1 hour, washed with ELISA wash buffer, and probed using an anti-mouse(IgG)-HRP conjugate (as above at 50 μL/well) or other appropriate antibody for 30 minutes, washed again with ELISA wash buffer, and then visualized by the addition of TM blue substrate (50 μL/well). After 10 minutes, the reaction is stopped by the addition of 1N H_2SO_4 (100 μL/well) and read on an ELISA plate reader set at 450 nm.

B. Immunogenicity of Particles

To assay the immunogenicity of particles, mice are immunized, IP, with 20 μg of particles in Freund's complete adjuvant, and then boosted at 4 weeks with 10 μg in Freund's incomplete adjuvant. Mice are bled at 2, 4, 6, and 8 weeks.

C.   Thermal Stability Protocol

Purified particles are diluted to a concentration of 1 mg/mL using 50 mM NaPO$_4$, pH 6.8 and sodium azide is added to a final concentration of 0.02% to prevent bacterial growth.   Particles are incubated at 37° C and aliquots are taken at a desired time point.   Samples are mixed with SDS-PAGE sample buffer (reducing) and run on 15% SDS-PAGE gels.   Gels are stained using Coomassie Blue, and then analyzed.

D: Analytical Gel Filtration
Analysis of Hybrid particles

Analytical gel filtration analysis of purified hybrid HBc particles is performed using a 25 mL Superose® 6 HR 10/30 chromatographic column (Amersham Pharmacia # 17-0537-01) and a BioCAD™ SPRINT Perfusion Chromatography System.   The UV detector is set to monitor both wavelengths of 260 and 280 nm.   The column is equilibrated with 3 column volumes (CV; about 75 mL) of buffer (50 mM NaPO$_4$, pH 6.8) at a flow rate of 0.75 mL/minute.

The particles to be analyzed are diluted to a concentration of 1 mg/mL using 50 mM NaPO$_4$, pH 6.8. 200 Microliters (μL) of the sample are then loaded onto a 200 μL loop and injected onto the column.   The sample is eluted from the column with 50 mM NaPO$_4$, pH 6.8 at a flow rate of 0.75 mL/minute. Integration of the 280 nm trace was carried out using BioCAD™ software (PerSeptive™) to provide the results.

Example 3: Determination of 280/260 Absorbance Ratios

Protein samples are diluted to a concentration of between 0.1 and 0.3 mg/mL using

phosphate buffered saline (PBS), pH 7.4. The
spectrophotometer is blanked, using PBS, and the
absorbance of the protein sample is measured at
wavelengths of 260 nm and 280 nm. The absorbance
value determined for a sample at 280 nm is then
divided by the absorbance value determined for the
same sample at 260 nm to achieve the 280/260
absorbance ratio for a given sample. The ratios were
obtained for several samples, including native
particles (HBc183), HBc particles truncated after
residue position 149 (HBc149), and several HBc
chimers that are identified elsewhere herein, are
shown below in Table 8. Full length particles ICC-
1559 are a preparation of the particles first
reported in Neirynck et al., (Oct 1999) *Nature Med.*,
5(10):1157-1163, whereas full length particles ICC-
1607 are similar particles in which the M2
polypeptide cysteines at polypeptide positions 17 and
19, (X_{17} and X_{19} of SEQ ID NO:9) were mutated to
serine residues.

Table 8

Particle Number	Full Length, (F) or C-Terminal Truncated, (T)	280/260 Absorbance Ratio
HBc183	F	0.84
HBc149	T	1.59
1438	T	1.57
1473	T	1.64

1475	T	1.04
1492	T	1.33
1559	F	0.68
1560	T	1.36
1590	T	1.51
1603	T	1.68
1604	T	1.40
1605	T	1.26
1607	F	0.73

Example 4:   Cysteine at the C-terminus
              of Truncated HBc Particle

A.   Addition of a Cysteine Residue
to the C-terminus of Hybrid HBc Particles

Using the polymerase chain reaction (PCR),
genes expressing hybrid HBc particles can be easily
mutated to introduce a cysteine or cysteine-
containing peptide to the C-terminus of an HBc chimer
that contains an added cysteine at the N-terminus.
For example, a PCR oligonucleotide primer that
encodes SEQ ID NO:256 can be used, in concert with a
suitable second primer, to amplify a hybrid HBc gene
and incorporate a cysteine codon between codon V149
and the stop codon. An exemplary construct is that
referred to as ICC-1492 that is discussed
hereinafter. See also, the preparation of V2.Pf1[N-
M2(17-24/C19S)] that is discussed hereinafter.

Hepatitis B core particles can be truncated
from 183 (or 185, depending on viral subtype) to 140
and retain the ability to assemble into particulate
virus-like particles. Many groups have used
particles truncated to amino acid 149 because amino

-124-

acid 150 represents the first arginine residue of the arginine-rich C-terminal domain.

Example 5:   Influenza M2 Constructs

Recently, Neirynck et al., (Oct 1999) *Nature Med.*, 5(10):1157-1163 and WO 99/07839 reported the fusion of the 24 amino acid extracellular domain of M2 to the N-terminus of full-length HBc particles (HBc183), lacking amino acid residues 1-4. A schematic representation of that construct referred to herein as IM2HBc is shown below in which the 24-mer is linked to the N-terminus of HBc.

IM2HBc

MSLLTEVETPIRNEWGCRCNDSSD-HBc(5-183)

SEQ ID NO: 256

In one illustrative preparation, the M2 epitope was inserted into the immunodominant loop of hepatitis B core and particles referred to as ICC-1475 were successfully expressed and purified using techniques discussed previously for such insertions and purifications. A mutated version of the M2 epitope, in which two cysteine residues at M2 native positions 17 and 19 were substituted by alanine residues, was also expressed in the immunodominant loop (ICC-1473 particles) and the resulting particles purified. These two particles are illustrated schematically below.

ICC-1475

HBc(1-78)-GI-SLLTEVETPIRNEWGCRCNDSSD-EL-HBc(79-149)

SEQ ID NO: 257

ICC-1473

HBc(1-78)-GI-SLLTEVETPIRNEWGARANDSSD-EL-HBc(79-149)-C

SEQ ID NO: 258

The ICC-1473 particle construct yielded approximately 7-fold more purified particles when compared with the native sequence (ICC-1475). It remains to be determined if the mutation of the cysteine residues alters protective potential of the particles. However, epitopes delivered on the immunodominant loops of HBc are usually significantly more immunogenic as compared to when they are fused to other regions (including the N-terminus), and resulting particles exhibit reduced anti-HBc immunogenicity.

Particles have also been prepared in which the M2 N-terminal 24-mer epitope was fused to the N-terminus of C-terminal truncated hepatitis B core particles. That construct (ICC-1438) also contained the N-terminal pre-core sequence (SEQ ID NO:259). A similar construct was prepared that contained a single cysteine residue at the end of the hybrid protein (ICC-1492), in this case immediately after Val-149 of the HBc gene. These constructs are shown schematically below.

ICC-1438

MG<u>I</u>SLLTEVETPIRNEWGCRCNDSSD<u>EL</u>LGWLWGI-HBc(2-149)

SEQ ID NO:259

ICC-1492

MG<u>I</u>SLLTEVETPIRNEWGCRCNDSSD<u>EL</u>LGWLWGI-HBc(2-149)-C

SEQ ID NO:260

It should be noted that to guard against translation initiation from the natural HBc initiator methionine, the codon for that residue was mutated to code for an isoleucine residue. Residues contributed by EcoRI (GI) and SacI (EL) restriction sites are underlined. The pre-core sequence is recited between the underlined EL residues and "-HBc(2-149)".

Analysis by SDS-PAGE as discussed elsewhere herein, showed that upon preparation, the ICC-1438 monomer construct was unstable (Lane 2) as compared to the ICC-1492 (Lane 3), with HBc-149 (Lane 1), ICC-1475 (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls on the SDS-PAGE gel in Fig. 10. The instability of the ICC-1438 monomers was not evident using analytical gel filtration of particles.

Both ICC-1475 (Fig.10, lane 4) and ICC-1473 (Fig.10, lane 5) were expected to have slightly lower molecular weights than ICC-1438 and ICC-1492, because the former two contain the M2 epitope inserted directly into the immunodominant loop and therefore lack the pre-core sequence (SEQ ID NO:259) present in ICC-1438 and ICC-1498. As expected, ICC-1492 was larger than ICC-1475 and ICC-1473; however, ICC-1438, which is identical to ICC-1492 save the C-terminal

cysteine residue, is clearly not larger than ICC-1475 and ICC-1473 due to an apparent cleavage.

A construct containing a M2 N-terminal extracellular sequence as discussed before linked to the HBc N-terminus (Domain I) or loop (Domain II) and also containing a cysteine residue at the C-terminus (Domain IV) of HBc is also contemplated.

To modify the amino-terminus of hybrid HBc particles containing immunodominant loop fusions to incorporate a cysteine residue, and minimal M2-derived sequence, a series of synthetic oligonucleotides are synthesized. To make V2.Pf1(N-M2(17-24/C17S), the oligonucleotides M2(17-24/C17S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 546 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which has been cleaved with the same two enzymes.

To make V2.Pf1[N-M2(17-24/C19S)], the oligonucleotides M2(17-24/C19S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene in vector V2.Pf1. The resultant 540 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which had been cleaved with the same two enzymes.

M2(17-24/C17S)-NcoI-F

```
    M  G  S  R  C  N  D  S  S  D  I  D  P  Y  K  E
.GGCGCCATGGGGTCTAGATGTAACGATTCAAGTGACATCGACCCTTATAAAGA
```

```
              F  G                    SEQ ID NO: 261
           ATTTCG                     SEQ ID NO: 262
```


M2(17-24/C19S)-NcoI-F

```
    M  G  C  N  D  S  S  D  I  D  P  Y  K  E  F  G
                    SEQ ID NO: 263
GCGCCATGGGGTGTAACGATTCAAGTGACATCGACCCTTATAAAGAATTTGG
                    SEQ ID NO: 264
```


Example 6:   HBc Chimer Molecules With and Without

           Both N- and C-Terminal Cysteine Residues

          A series of HBc chimer molecule-containing
particles was prepared that contained residues 1-24
of the influenza A, M2 protein peptide-bonded at or
near the N-terminus of HBc whose C-terminus was
truncated at residue 149.   The component chimeric
protein molecules contained different N-terminal
sequences that included an M2 sequence or variant,
and some contained a C-terminal cysteine residue.

          All purified particles listed in Table 9,
hereinafter, were analyzed by analytical size
exclusion chromatography to assess the retention of
particulate structure following purification.
Particles designated ICC-1603, which contain no N-
terminal cysteine residues, displayed evidence of
disassembly back to sub-particulate structures (Fig.
3) because the protein eluted in the 1500 second

range (particles elute at approximately 1000 seconds).

Similar analysis of particles ICC-1590, which are similar to ICC-1603 ICC-particles except for the mutation of two serine residues to cysteine residues in the N-terminal M2 sequence, revealed that that construct remained particulate following purification, with elution occurring at around 1000 seconds, which is typical for a hybrid particle (Fig. 4). There was no evidence of disassembly for ICC-1590 particles.

Analysis of ICC-1560 particles, whose chimer protein also has two N-terminal cysteine residues, revealed that it too was particulate following purification, although it did exhibit some degree of disassembly (Fig. 5), suggesting that the stabilization was not quite as robust as it was for ICC-1590 particles. Comparison of the N-terminal configurations of ICC-1590 and ICC-1560 particles (Table 11, hereinafter), shows that the relative position of the two cysteine residues in ICC-1560 particles is shifted by 3 amino acid residues relative to ICC-1590 particles via the deletion of three amino acid residues (DEL), indicating that the cysteine residues may be required to be a minimal distance from the start of the core gene to enable optimal cross-linking.

Example 7: Particles With an M2 or M2 Variant
                Sequence and A C-Terminal Cysteine Residue

ICC-1603 particles were shown in Fig. 3 to rapidly disassemble following purification. The HBc chimer molecules that comprise ICC-1605 particles are

similar to those of ICC-1603 particles, except that
the ICC-1605 component chimer molecules have a single
C-terminal stabilizing cysteine. A plasmid was made
to direct the expression of ICC-1605 particles to
investigate if the addition of a C-terminal cysteine
residue to ICC-1603 particles could impart greater
stability on the particle. Following purification,
ICC-1605 particles were analyzed using analytical size
exclusion chromatography (Fig. 6).

The results of this study demonstrated that
particle stabilization was more complete than for the
ICC-1603 particles, but incomplete compared to ICC-
1590 particles, which contains two amino-terminal
cysteine residues and no C-terminal stabilizing
cysteine. Although a significant amount of ICC-1605
remained particulate, there was evidence of a
heterogeneous mixture of sub-particulate structures
that eluted over a broad range. These observations
suggest that for this hybrid particle (ICC-1603),
C-terminal stabilization as found in ICC-1605
particles was less complete than for the N-terminal
stabilization found in ICC-1590 particles.

To investigate the compatibility of combined
amino and carboxyl-terminal cysteine stabilization of
hybrid particles, an expression plasmid was
constructed to direct the expression of ICC-1604
particles. The component chimer molecules of ICC-1604
particles contain both the two amino-terminal
stabilizing cysteine residues present in a native M2
polypeptide sequence (as in ICC-1590) as well as a C-
terminal stabilizing cysteine (as in ICC-1605
particles). Analysis of ICC-1604 particles showed
that they retained a homogeneous particulate state
following purification (Fig. 7), indicating that the

two stabilizing methods are complementary and can be used in concert with each other.

Alternative linker sequences between the N-terminus of HBc and the N-terminal cysteine residues were investigated using particles ICC-1438 and ICC-1492. Both of these particles contain the amino acid sequence ELLGWLWGIDI (SEQ ID NO:265) between the M2 fusion and amino acid D4 of HBc. The C-terminal nine amino acid residues of that sequence are derived from amino acids -6 of the HBc pre-core sequence to amino acid I3 of HBc, with the initiator codon of HBc mutated to an isoleucine to prevent translation initiation from this position, which would compromise the study. The HB pre-core sequence includes a cysteine at position -7.

These particles differed only in the fact that the ICC-1438 component chimer molecule terminated at position 149 of HBc, whereas the ICC-1492 component chimer molecule terminated at 149 of HBc and contained a terminal cysteine at position 150 relative to the HBc of SEQ ID NO:1. When analyzed by analytical gel filtration, using an alternative but similar method to that discussed before, whereby particles elute at approximately 10 minutes, both constructs were shown to be particulate following purification (ICC-1438 in Fig. 8 and ICC-1492 in Fig.10). This study demonstrated the compatibility of amino- and carboxyl-terminal cysteine stabilization of truncated particles, and the tolerance of substantial variability in the amino acid sequence and distance between the N-terminal cysteine residues and start of the HBc gene.

Table 9

Construct Name	N-terminal Fusion	HBc N-term Start	Residues Between M2 and HBc	C-term End	Bound Nucleic Acid	C-term Cysteine Stab
ICC-1560	M2 (1-24)	D4	None	149	No	No
ICC-1603	M2 (1-24) (2C>2S)	D4	EL	149	No	No
ICC-1590	M2 (1-24)	D4	EL	149	No	No
ICC-1604	M2 (1-24)	D4	EL	149	No	Yes (C150)
ICC-1605	M2 (1-24) (2C>2S)	D4	EL	149	No	Yes (C150)
ICC-1438	M2 (1-24)	D2	ELLGWLWGI	149	No	No
ICC-1492	M2(1-24)	D2	ELLGWLWGI	149	No	Yes (C150)

Table 10, below, shows an alignment that illustrates the configuration of the N-termini of HBeAg, and particles designated ICC-1590, ICC-1560, ICC-1603, ICC-1604 and ICC-1605. Sequences are aligned according to amino acid residue position 4 from the N-terminus of HBc of SEQ ID NO:1 that is shared by all constructs. N-terminal cysteine residues, when present, are underlined.

Table 10

Construct Name	Sequence	SEQ ID NO
HBeAg	SKL_C_LGWLWGMDID	266
ICC-1590/ICC-1604	MSLLTEVETPIRNEWG_C_R_C_NDSSDELD	267
ICC-1560		

MSLLTEVETPIRNEWGCRCNDSSD   268

ICC-1603/ICC-1605

      MSLLTEVETPIRNEWGSRSNDSSDELD   269

ICC-1438/ICC-1492

 MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGIDID   270

     Table 11, below, provides a tabulation of the results in which stability was assessed for particles containing an N-terminal influenza A M2 sequence or variant contemplated herein. As is seen, stable particles have been prepared from HBc chimer molecules that contain an N-terminal cysteine residue at a position of minus 14 (-14) relative to the N-terminus of the HBc sequence of SEQ ID NO:1 to about the N-terminus itself.

Table 11

Construct Name	Amino Acids Between HBc D4 and N-terminal Cysteine Residues		C-terminal Cysteine Stabilization	Stable Particle Formed
	Cys 1	Cys 2		
HBeAg	-	9	No	No
ICC-1603	-	-	No	No
ICC-1605	-	-	Yes	Yes/No
ICC-1590	9	7	No	Yes
ICC-1604	9	7	Yes	Yes
ICC-1560	6	4	No	Yes
ICC-1438	18	16	No	Yes
ICC-1492	18	16	Yes	Yes

Each of the patents and articles cited herein is incorporated by reference. The use of the article "a" or "an" is intended to include one or more.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.